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New signaling pathways involved in mast cell activation and cell membrane repair

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This thesis summarizes the results obtained during my six-year stay at Department of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic. All experimental data are compiled into four articles, three of them have been published and the fourth one has been submitted for publication. The first article, "Regulation of Ca^{2+} signaling in mast cells by tyrosine-phosphorylated and unphosphorylated non-T cell activation linker, NTAL" was published in The Journal of Immunology 2007, 179: 5169 –5180. Second, "Tetraalkylammonium derivatives as real-time PCR enhancers and stabilizers of the qPCR mixtures containing SYBR Green I" was published in Nucleic Acids Research 2008, 36:1-10. Third, "Changes in GM1 ganglioside content and localization in cholestatic rat liver" was published in Glycoconjugate Journal 2007, 24:231-241. Fourth, "Vacuolin-1-modulated exocytosis and cell resealing depend on cell origin and mode of activation" was submitted in EMBO reports".

All of these articles are with multiple authors, reflecting involvement of demanding methods and approaches. To indicate my contribution to these studies I mentioned my contribution to each of them in brief summary preceding individual papers in the Results section.

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List of abbreviations

ATP	Adenosine tri-phosphate
BCR	B cell receptor
BMMCs	Bone marrow-derived mast cells
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
$[Ca^{2+}]_i$	Concentration of free intracellular calcium
Cbp	Csk-binding protein
CD	Cluster of differentiation
Csk	C-terminal Src kinase
CTMCs	Connective tissue mast cells
DAG	Diacylglycerol
EGFR	Epidermal growth factor receptor
Erk	Extracellular-signal regulated kinase
FcεRI	Type I receptor for IgE
Gab-2	Grb2 associated binder 2
Gads	Grb2-related adapter downstream of Shc
GEF	Guanine nucleotide exchange factor
GEMs	Glycosphingolipid-enriched microdomains
Grb2	Growth factor receptor-bound protein 2
IL	Interleukin
IP3	Inositol 1,4,5 triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
JNK	Jun N-terminal kinase
LAB	Linker for activation of B cells
LAT	Linker for activation of T cells
LPS	Lipopolysaccharide
Lyn	Lck/Yes-related novel tyrosine kinase
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemoattractant protein 1
MIP-1 α	Macrophage inflammatory protein 1 α
MMC	Mucosal mast cells
NFAT	Nuclear factor of activated T cells
NRK cells	Normal rat kidney cells
NTAL	Non-T cell activation linker
PAF	Platelet-activating factor
PAG	Phosphoprotein associated with glycosphingolipid-enriched microdomains
PCMC	Peritoneal cell-derived mast cells
PCR	Polymerase chain reaction
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PLCγ	Phospholipase Cγ
PTB	Phosphotyrosine binding
PTK	Protein tyrosine kinase
qRT-PCR	Quantitative real-time PCR
RBL	Rat basophilic leukemia
SCF	Stem cell factor
SH	Src homology
SHIP	SH2-domain containing inositol polyphosphate 5-phosphatase
SHP	SH2-domain containing phosphatase
SLO	Streptolysin-O
SLP-76	SH2-domain containing leukocyte protein of 76 kDa
Sos	Son of sevenless
Syk	Spleen tyrosine kinase
TAA-Cl	Tetraalkylammonium chloride
TCR	T cell receptor

TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TRAP	Transmembrane adaptor protein
ZAP-70	ζ associated protein of 70 kDa

CONTENTS

1. INTRODUCTION

1.1 Mast cells and basophils

Immune responses in host defense and diseases depend on the cooperation of various cells, both circulating and tissue resident. Mast cells and basophils have been most extensively studied in relation to the IgE-associated immune responses against parasites and mainly as the early effectors of allergic reactions. Recently, however, mast cells have been repeatedly shown to be critically involved in defense against bacterial infections and evidence for their participation in early responses to viral and fungal pathogens is growing. Nevertheless, nowadays the IgE-mediated hypersensitivity still remains the major scope of most research conducted on mast cells and basophils.

1.2 Ontogeny and subsets

Mast cells and basophils have many features in common, but they also differ in several functional and developmental aspects. Like other leukocytes, mast cell and basophils are derived from a common progenitor hematopoietic stem cell. Basophils mature in bone marrow and enter circulation as terminally differentiated cells (Wedemeyer et al., 2000). In contrast, mast cells exit bone marrow as immature committed progenitors that subsequently migrate into various tissues, where they undergo maturation (Gurish and Austen, 2001; Gurish et al., 1995). Mast cell progenitors present in circulation and as a large pool in the intestinal mucosa bear typical surface characteristics, c-kit^{+}CD34^{+}CD13^{+}FcεRI^{-}. Maturation of mast cell progenitors depends on different situation within a tissue (Friend et al., 1996; Gurish et al., 1995; Stevens et al., 1994); interestingly the committed progenitors do not develop into mature mast cells in the absence of inflammation (Gurish and Austen, 2001). Stem cell factor (SCF) and c-kit, a receptor-type tyrosine kinase interaction is indispensable for mast cell development or maturation. Introduction of mutations in *c-kit* gene leads to defects in mast cell development and complete loss of functional mast cells. Knock-out studies in mice have been very useful in assessing the function of mast cells in vivo. Adoptive transfer of genetically compatible in vitro derived mast cells compensated the knock-out effect (Galli et al., 2005).

The number of basophils, on the other hand are regulated mainly by interleukin 3 (IL-3). IL-3 is regulator but not the vital factor for propagation. Absence of IL-3 doesn't cause a complete loss of basophils, but rather inhibits the expansion of basophil population, for instance during parasitic infection (Galli, 2000).

Accordingly, culturing of mouse bone marrow derived mast cells (BMMCs) in vitro in the presence of IL-3 results in a uniform population of immature mucosal mast cells (MMC) (Lantz and Huff, 1995; Nakahata et al., 1986; Tsuji et al., 1990). Moreover, nerve growth factor and SCF can induce proliferation of MMCs and promote their maturation to acquire some characteristics of connective tissue mast cells (CTMCs) (Ducharme and Weis, 1992; Dvorak et al., 1994; Lantz and Huff, 1995; Smith et al., 1994; Tsai et al., 1991). Historically two main phenotypes have been reported in rodents. T cell independent CTMCs, which contain heparin inside the granules and T cell dependent MMCs holding mainly mono- and di-sulfated chondroitin specific proteases, even more subpopulations can be distinguished in vivo. It was also demonstrated that mast cells can alter their phenotype depending on micro environmental factors (Lutzelschwab et al., 1997). Also in humans two main populations of mast cells are recognized, though in contrast to rodents based preferentially on the content of diverse proteases. Most human mast cells present in connective tissues store in the cytoplasmic granules chymase and tryptase, whereas mast cells located in the tissues express only tryptase and lack chymase (Feger et al., 2002). In both human as well as rodent MMC maturation is T-cell dependent, while CTMCs are not affected by T cells in their development (Austen and Boyce, 2001). Compared to basophils mast cells are long lived cells residing in the tissues, where they can divide under certain circumstances. Basophils, however, are normally present in very low numbers in the circulation and survive only several days. They are usually recruited to peripheral tissues during inflammation. Both mast cells and basophils are highly granulated, but unlike other granulated cells, they release the content of secretory vesicles in a single stimulatory event.

1.3 Degranulation, functions in innate and acquired immunity, receptors and their expression

1.3.1 Degranulation

Upon stimulation by various triggers, mast cells and basophils are able to release the content of preformed cytoplasmic granules as well as to start de novo synthesis of eikosanoids and several cytokines (Blank and Rivera, 2004). Secretory granules of both mast cells and basophils contain preformed mediators such as biologically active amines, namely histamine and serotonin in rodents, as well as proteoglycans and neutral proteases. Moreover, several lipid mediators like prostaglandin D_2 in mast cells and leukotrine C_4 in both mast cells and basophils, are formed upon stimulation via IgE. Such stimulated mast cells have been recognized to be the source of several de novo synthesized cytokines, namely IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and several chemokines namely macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1. IL-4 and IL-13 are the main cytokines reported in basophils (Brunner et al., 1993; Li et al., 1996b; MacGlashan, Jr. et al., 1994).

1.3.2 Mast cells in Innate immunity

Mast cells are strategically located at the host-environment interface with proved function to provide an early defense against invading pathogens. Lack of mast cells in c-Kit knockout mice resulted in compromised innate defense mechanism (Echtenacher et al., 1996). Another body of evidence for mast cells involvement in innate immunity came from studies in which repetitive treatment of mice with SCF, which is known to increase mast cell numbers and to modulate their functions in normal mice, can enhance survival of mice with artificially induced bacterial peritonitis (Maurer et al., 1998). Mast cells express many different adhesion and receptor molecules and may efficiently respond to pathogen-associated signals. First, mast cells express on their surface complement and Fc receptors, and therefore they can directly recognize and attach to microbes coated with complement components or opsonized by antibodies (Gommerman et al., 2000; Prodeus et al., 1997). Second, the C3a and C5a have been identified to be chemotactic for mast cells. Thus mast cells can be selectively recruited to sites of complement activation and enhance their participation in host defense (Marshall, 2004). Third, a

wide range of Toll-like receptors (TLRs) are expressed in mast cells. Rodent cells normally produce pro-inflammatory cytokines, TNF-α and IL-6 without concurrent degranulation, upon stimulation with bacterial lipopolysaccharides (LPS). However, mast cells deficient in TLR-4 were unable to respond to LPS, indicating that TLR-4 engagement is crucial for this activation. In addition, both rodent and human mast cells have been shown to express TLR-9 and to synthesize several cytokines in response to CpG-containing oligonucleotides (Marshall, 2004). Although the range of various mediators produced by mast cell is enormous, mast cells response could also be highly specific. The cytokine and other mediator profile secreted by activated mast cells depends on three types of pathogen-associated stimuli they encounter. In the context of pathogen defense the TNF-α and leukotrien B_4 (LTB_4) were revealed as the main mediators secreted by mast cells (Malaviya et al., 1996). TNF-α which functions to increase the expression of adhesion molecules on endothelium, together with LTB4, a potent neutrophil chemoattractant ensure recruitment of neutrophils to the place of bacterial invasion. Nevertheless mast cells are also able to process and present microbial antigens of T-cells through MHC.

1.3.3. Mast cells in Acquired immunity

The best studied role of mast cells and basophils as effectors of acquired immunity is their involvement in IgE-associated response. This response may be protective (anti-parasite immunity) or deleterious (anaphylactic reactions).

1.3.3.1 Anti-parasite immunity

It has been observed that the parasitic infections are associated with elevated numbers of circulating basophils and eosinophils along with significantly increased levels of serum IgE and up-regulated counts of mast cells and basophils in the effected tissue (Wedemeyer et al., 2000). It now seems obvious that mast cells, basophils and eosinophils are critical components of broader T_H2 polarized response in the host defense against parasites. With neutralizing antibodies to c-Kit ligand, SCF, or using mast cell-deficient mice strains, which lack functional copy or either c-Kit ligand or SCF, it was shown that mast cells are essential for immune response to *Trichinella spiralis*. Mast cell deficient mice and or SCF-neutralizing antibody treated animals exhibited ineffective clearance of the intestinal worms (Donaldson et al., 1996; Grencis et al., 1993). Recently the role of up regulated serum IgE levels was studied in mice with targeted disruption

of IgE H chain gene. Even though the mast cell response in the intestine in IgE negative mice was comparable to their wild-type littermates, serum level of mouse mast cell protease 1 was lower and the elimination of *T. spiralis* was delayed in IgE deficient mice. Moreover the number of larvae present in the skeletal muscles was almost two-fold higher and the fraction of necrotic larvae reduced in IgE negative mice. Thus IgE clearly contributes to the parasite clearance at two different stages in its life cycle (Gurish et al., 2004). While investigating the immune response to the same parasite, *T. spiralis*, it was observed that mucosal mast cells are directly responsible for enhancement of epithelial paracellular permeability and expulsion of parasite from the intestine. Furthermore, mice deficient in mast cell specific protease do not develop increased intestinal permeability upon *T. spiralis* infection and fail to expel their parasite burden (McDermott et al., 2003).

1.3.3.2 Immunopathology and hypersensitivity

Several pathogen-associated situations have been reported when FcεRI was crosslinked independently of antigen recognition. The HIV glycoprotein 120 (gp 120) can interact with the V_H3 domain of the IgE molecule and thereby induce mediator release from mast cells and basophils. In this regard the T_H2 polarizing IL-4 and IL-3 secreted by mast cells and basophils may be involved in the T_H2-cytokine pattern observed in early phase of HIV infection (Patella et al., 2000). Protein Fv, an endogenous protein released into the intestinal tract in patients with viral hepatitis, can induce degranulation of mast cells and basophils and cytokine production by identical mechanism (Patella et al., 1998). Such proteins are sometimes referred as “superallergens”, in parallel to superantigens.

1.3.3.3 Type I allergic reactions

As mentioned above, mast cells are well established as effectors of innate and IgE-dependent responses. However, mainly due to the wide array of inflammatory mediators they express, mast cells also substantially participate in pathology of allergic diseases and anaphylactic reactions. Allergic reactions arise when IgE antibody is produced in response to otherwise harmless antigen, allergens, present in environment such as pollen, dust and some foods, fungal spores and many others. Upon encounter of particular allergen the specific IgE bound to the surface of FcεRI triggers the activation and leads to release of inflammatory mediators. Although the

precise mechanisms leading to allergy are not understood completely it seems that certain antigens and routes of their presentation to the immune system induce T_H2 polarized IgE response. Furthermore low concentration of antigen or low occupancy of the receptor with IgE potently favors production of lymphokines known to promote allergic inflammation.

Mediators released from activated mast cells are the main effectors of allergy associated pathophysiological conditions. Thus mast cells are essential for immediate phase reaction, which includes enhanced vascular permeability and oedema associated with anaphylactic reactions in mice as well as in humans (Wedemeyer et al., 2000). Histamine, a short lived vasoactive amine, is responsible for an immediate increase in vessel permeability. Through this process, histamine together with TNF-α activates endothelial cells to express adhesion molecules promoting the accumulation of inflammatory leukocytes and lymphocytes in the tissue. Additionally mast cell proteases, chymase, tryptase activate matrix matalloproteinases and thus contribute to tissue damage. Based on experiments in murine model of allergy and asthma it was clearly demonstrated that T_H2 cytokines such as IL-4, IL-5, IL-9 and IL-13 play an essential role in pathogenesis of allergic inflammation. IL-4 promotes adhesion of circulating eosinophils to endothelial cells along with IL-5, which attracts them to target tissue. IL-13 induces mucus hyperproduction and together with IL-4 stimulates fibroblast growth and the synthesis of extracellular matrix protein.

IL-4 which is known to control B cell class switching to IgE producing plasma cell and T cell maturation in to IL-4 and IL-5 producing TH2 cells, seems to positively affect proliferation of mature human mast cells and enhances mediator release. In this way, IL-4 serves as a positive feed-back in the allergic inflammation process (Bischoff et al., 1999). Thus, mast cells are responsible for virtually all of the augmented vascular permeability and oedema associated with anaphylactic reactions.

1.4 FcεRI receptor

Although there is considerable evidence showing that some pathophysiological consequences of allergic reactions may occur in the absence of IgE, typical anaphylactic reactions are IgE-dependent. Most importantly, experiments with FcεRI-deficient mice have demonstrated that, for

IgE-mediated anaphylactic reactions the presence of FcεRI is essential (Dombrowicz et al., 1993). In terms of molecular structure and general mechanisms of early signaling events, FcεRI belongs to a family of immune receptors named multichain immune recognition receptors.

1.4.1 FcεRI structure

The originally described rodent FcεRI forms a tetrameric structure composed of one α chain, one β chain and two identical, disulfide-bridged γ chains (Blank et al., 1989). These components are maintained in the plasma membrane through a combination of electrostatic and hydrophobic interactions (Kinet et al., 1985). Cell surface expression of rodent FcεRI requires pre-assembly of all three types of polypeptides, α, β and γ, in the endoplasmic reticulum (ER). Human FcεRI can be expressed as either tetrameric ($\alpha\beta\gamma_2$) or trimeric ($\alpha\gamma_2$) structure. In fact these two types of FcεRI differ in their cellular distribution. Tetrameric FcεRI is strongly expressed on mast cells and basophils, probably also on platelets and a subset of eosinophils. Trimeric FcεRI has been found on monocytes and dendritic cells. Given the differences in expression and in signal transduction, it seems likely that the trimeric structure on antigen presenting cells (APC) serves a distinct function involved in antigen uptake (Kinet, 1999).

The FcεRI α chain is a member of the immunoglobulin superfamily and is responsible for the high affinity and specificity for IgE. It consists of a short intracellular portion, one transmembrane helix and two extracellular immunoglobulin-like glycosylated domains. The extracellular domains D1 and D2 adopt a highly bent arrangement creating two interaction sites for Cε3 domains of IgE. Thus, the receptor binds the IgE molecule asymmetrically and strictly at 1:1 stoichiometry (Garman et al., 2000). Additional gain in stability of the FcεRI-IgE complex results from the interaction of Cε2 domain of IgE with both the α chain and Cε3 domain (McDonnell et al., 2001) carbohydrates attached to the α chain cover the sides of the receptor and do not contribute to IgE binding, but they are important for proper folding in the ER (Kinet, 1999).

The β chain spans the plasma membrane four times, with both N- and C-terminal tails remaining intracellularly. Although the N-terminal tail may be involved in signal transduction, its exact role in mast cell physiology is yet to be elucidated. The C-terminal tail has been proposed to mediate

constitutive binding of a key signal transduction molecule - Src family kinase Lyn. Furthermore it contains one immunoreceptor tyrosine based activation motif (ITAM) which upon phosphorylation, recruits SH2 domain containing signaling molecules, especially Lyn. The same β chain is also a part of the low affinity IgG receptor complex, FcγRIII (Kinet, 1999).

The γ chain is a member of the γ/ζ/η family of immunoreceptor subunits and consists of a single transmembrane region and a cytoplasmic tail. The γ chain and other members of this family can be found in homo or heterodimers complexed to various receptors of leukocytes, and serves as modular signal-transducing units. In the case of FcεRI, γ forms a disulfide linked homodimer, where each of the chain contains one ITAM. Similarly to its relatives, phosphorylated γ chain ITAM binds protein tyrosine kinases of Syk/ZAP-70 family (Jouvin et al., 1995).

1.4.2 Regulation of expression

The surface expression level of FcεRI is regulated mainly by the concentration of circulating IgE and in part also by cytokines like IL-3. The effect of IgE on FcεRI expression was first demonstrated in rat basophilic leukemia (RBL) cells, clone 2H3. When RBL-2H3 cells were cultured in the presence of IgE, the amount of FcεRI expressed on the surface increases approximately twice (Furuichi et al., 1985). Even more dramatic effect of IgE was observed in IgE-deficient mice. In IgE null mice the level of FcεRI expression on peritoneal mast cells is significantly reduced, while it becomes markedly up regulated upon exposure to IgE (Yamaguchi et al., 1997). Binding of IgE to receptor α subunit is responsible for up regulation, apparently due to stabilization and thus accumulation of the receptor at the plasma membrane (Borkowski et al., 2001). Moreover IgE was shown to have an anti-apoptotic effect on mast cells, most likely by its ability to induce the production and secretion of cytokines by mast cells. The capacity of various IgE molecules (clonal variants) to trigger cytokine production in the absence of antigen differs widely. Thus the survival effects of a particular IgE correspond to the strength of the evoked response. The effects of IgE on mast cell survival and FcεRI expression were also studied in FcεRIγ-negative mast cells isolated from transgenic mice expressing wild type or ITAM mutant γ subunit. It was shown that although IgE-mediated mast cell survival depends on

functional FcεRI-γ subunit ITAM, the up regulation of cell surface FcεRI expression is independent of this motif (Sakurai et al., 2004).

In relevance to allergic diseases the human gene for FcεRI-β chain has been identified as a polymorphic region with possible significance to atopy. Indeed, several polymorphisms have been detected in the β chain, some with strong genetic linkage to allergy (Sandford et al., 1993). As β chain serves as an amplifier of surface expression and signal transduction through FcεRI these studies may provide a promising step in understanding a potential immunologic dysregulation that may contribute to the development of atopy.

1.4.3 Signal transduction

Intracellular signaling from FcεRI links the recognition of antigen or allergen by specific IgE to effector functions, such as degranulation and cytokine synthesis. Due to high affinity of the receptor, mast cells can be loaded with monomeric IgE which does not rely on recognition of preformed IgE-antigen complexes. Thus the original concept of signaling suggests that IgE binding is insufficient to induce intracellular signaling, but mechanical cross linking of IgE-FcεRI complexes on the cell surface is required. In support of this, the binding of IgE itself was not found to be associated with a conformational change in the receptor that could be transduced into the interior of the cell (Garman et al., 1999). However, in addition to the well demonstrated fact of surface expression regulation, which is independent of de novo protein synthesis, two studies have showed that binding of monomeric IgE to FcεRI has substantial effects on intracellular events and may increase survival of growth factor deprived mast cells in vitro, as well as induce cytokine secretion (Asai et al., 2001; Kalesnikoff et al., 2001). Thus elevated IgE concentrations might not only promote surface density of the receptor but also increase mast cell numbers.

1.4.4 Early signaling events

The cytoplasmic portions of β and γ subunits of FcεRI contain ITAMs. ITAM is a structural motif that has a consensus sequence YXX(L/I/V)X_{6-8}YXX(L/I/V), where X represents any amino acid. Upon antigen crosslinking of the FcεRI-bound IgE, tyrosine residues present in ITAMs become phosphorylated by Src family protein tyrosine kinase (PTK) Lyn (Eiseman and

Bolen, 1992; Vonakis et al., 1997). Lyn is essential for mediating the tyrosine phosphorylation of FcεRI as determined in experiments with Lyn deficient BMMCs. However the precious mechanism of receptor phosphorylation is yet to be elucidated. So far two alternative models have been proposed. First, a small fraction of Lyn kinase was detected to be constitutively associated with FcεRI in the resting cells (Wilson et al., 2000; Pribluda et al., 1994). After multivalent antigen is bound to the FcεRI-associated IgE, FcεRIs are brought close to each other and the cytoplasmic subunits can be trans-phosphorylated by Lyn bound to the juxta-receptor, while the membrane anchoring of Lyn serves only to facilitate this interaction (Kovarova et al., 2001; Pribluda et al., 1994). Second model postulates that specialized lipid environments facilitate the interaction between the aggregated FcεRI and Lyn (Holowka and Baird, 2001; Young et al., 2003; Dráber and Draberova, 2002) It is thought that ligand-formed receptor aggregates move to lipid rafts (also referred as glycosphingolipid enriched microdomains, GEMS) (Brown and London, 1998; Simons and Ikonen, 1997) or membrane rafts (Pike, 2006) containing Lyn, since Lyn due to its N-terminal dual acylation portions into the membrane rafts (see 1.4.4). Thus Lyn can phosphorylate receptor subunits brought to the kinase vicinity. Phosphorylated ITAM on β chain serves as docking site for more Lyn kinase whereas phosphorylated γ subunit ITAMs recruit cytoplasmic kinase Syk to plasma membrane (Minoguchi et al., 1994). Binding of Syk to the phosphorylated ITAMs causes a conformational change in the enzyme leading to Lyn-dependent phosphorylation and activation of the kinase (El-Hillal et al., 1997). Using Syk-deficient primary murine mast cells it was demonstrated that Syk is essential for FcεRI-dependent signal transduction, since mast cells derived from Syk-negative mice fail to degranulate and synthesize cytokines (Costello et al., 1996). Later, another Src family PTK, Fyn, was identified to be engaged upon FcεRI aggregation (Parravicini et al., 2002). Fyn functions to phosphorylate scaffolding protein Gab2, which is important for FcεRI-triggered activation of phosphatidylinositide 3 kinase (PI3 Kinase) in mast cells (Gu et al., 2001). Lyn and Fyn seem to have non redundant roles in mast cell signaling.

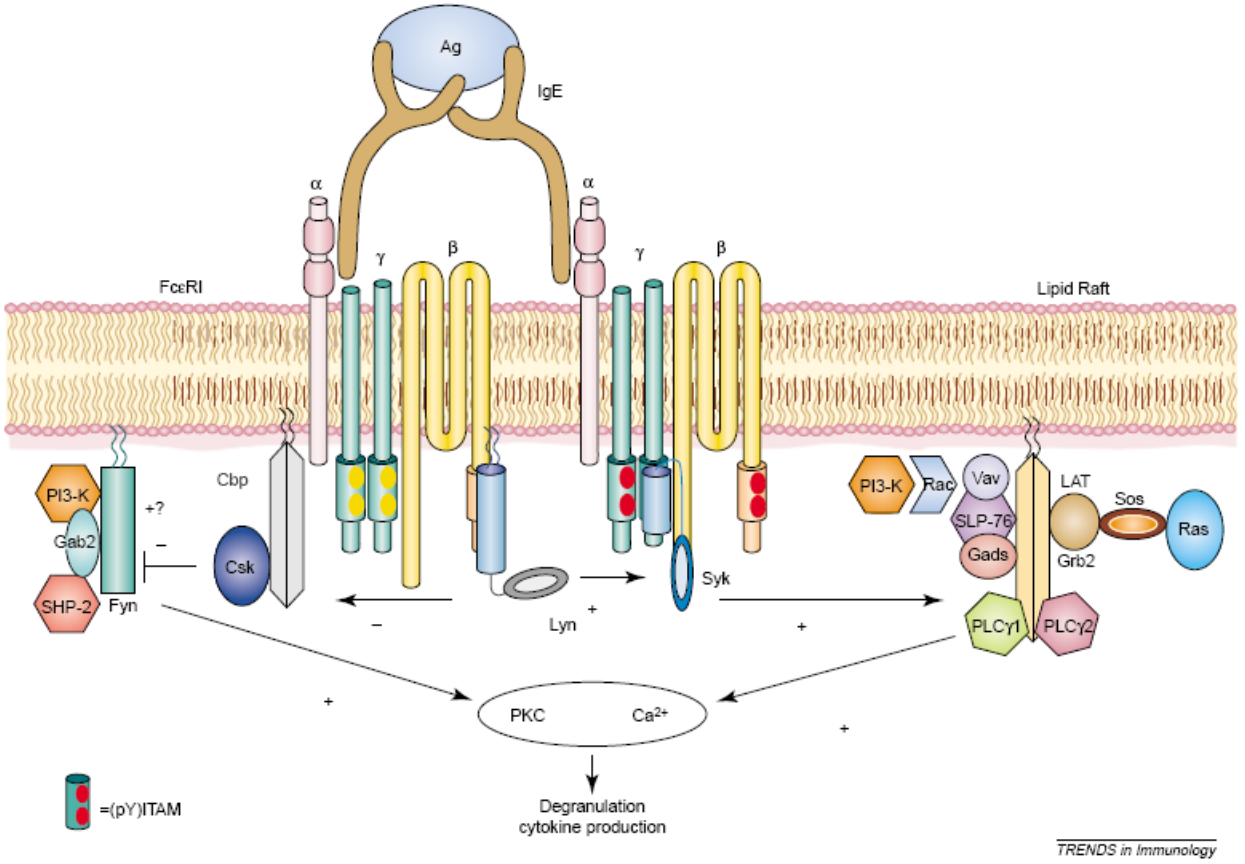

Fig 1. A model explaining structure and functional coupling of the high affinity receptor for immunoglobulin E (FcεRI) to early signaling events (Blank and Rivera, 2004). For detailed description see main text.

In Lyn-deficient mast cells phosphorylation of FcεRI ITAMs as well as other proteins is impaired and the rise of cytoplasmic Ca^{2+} concentration following FcεRI engagement is delayed. However the release of cytoplasmic granules from these cells remains unaffected. Quite the opposite is true for Fyn-negative mast cells. These cells show normal β and γ receptor subunit phosphorylation and intracellular Ca^{2+} mobilization, but degranulation is markedly decreased (Parravicini et al., 2002). Most recently negative regulatory role of Lyn in mast cells has been in part elucidated. Lyn-negative BMMCs show higher kinase activity of Fyn, resulting in increased degranulation (Hernandez-Hansen et al., 2004; Odom et al., 2004). Furthermore, the tyrosine phosphorylation of adaptor protein PAG (protein associated with GEMs), alternatively named Csk (C-terminal Src kinase), a negative regulator of Src PTKs, seems to block in a negative feedback loop resulting in mast cell hyper responsiveness of $Lyn^{-/-}$ cells (Odom et al., 2004).

Therefore, both Lyn and Fyn are necessary for FcεRI mediated response. Downstream of Lyn and Fyn multiple adaptor proteins are involved to propagate FcεRI initiated signal.

1.4.5 Adaptor proteins

Adaptor proteins function to organize molecular components required for signaling and coordinating receptor-stimulated responses. Adaptors participate in both positive and negative regulation. They do not exert any enzymatic or transcriptional activity but direct protein-protein and protein-lipid interactions. These inter molecular interactions are mediated by various structural domains. Thus, Src-homology (SH) 2 and phosphotyrosine binding (PTB) domains recognize phosphorylated tyrosines in the context of adjacent amino acid residues. Moreover, SH3 domain binds to its ligands via proline rich region, pleckstrin homology (PH) domain associates with specific membrane phospholipids (Myung et al., 2000). Adaptor proteins can be basically divided in to two groups: cytoplasmic and transmembrane adaptor proteins (TRAPs). To the first type of adaptors belong for instance growth factor receptor-bound protein 2 (Grb2), Grb2 associated binder 2 (Gab2), SH2 domain containing leukocyte protein 76 kDa (SLP-76). TRAPs are represented by linker for activation of T cells (LAT), non T cell activation linker (NTAL), also known as linker for activation of B cells (LAB) or LAT2, and PAG/Cbp. Grb2 is ubiquitously expressed adaptor protein originally identified by its ability to bind to the tyrosine-phosphorylated tail of the epidermal growth factor receptor (EGFR) (Lowenstein et al., 1992). Grb2 consists of one SH2 domain flanked by SH3 domains (Koch et al., 1991). It was determined in earlier studies in T cells and B cells that following immunoreceptor stimulation, association of Grb2 with the exchange factor SOS recruits to the plasma membrane (Harmer and DeFranco, 1997; Li et al., 1996a). This allows SOS to activate membrane-bound small G-proteins Ras. Interaction of Grb2 with SOS was also observed in mast cells; upon FcεRI engagement SOS activates Ras via Shc-Grb2-SOS (Jabril-Cuenod et al., 1996). However, more recently another mechanism leading to activation of Ras pathway in mast cells has been proposed. It was shown that Syk/PKCβ1-dependent pathway results in activation of Ras/Erk signaling cascade. Upon FcεRI stimulation, PKCβ1 is recruited to the plasma membrane via diacylglycerol (DAG) and acid phospholipids, and is subsequently phosphorylated by Syk at Y662. Tyrosine phosphorylated C-terminus of PKCβ1 brings Grb2-SOS complex to the vicinity of Ras and increases GTP bound form of Ras (Kawakami et al., 2003).

Another cytoplasmic adaptor protein SLP-76 is predominantly expressed in hematopoietic cells, notably in T cells and myeloid cells (Jackman et al., 1995). SLP-76 comprises multiple N-terminal tyrosine phosphorylation sites, as central proline rich region, and a SH2 domain on its C-terminus. In T cells SLP-76 is rapidly tyrosine phosphorylated by the protein tyrosine kinase (PTK) ZAP-70 after T cell antigen receptor (TCR) engagement (Bubeck et al., 1996). In RBL-2H3 cell line SLP-76 was recognized as substrate of FcεRI stimulated PTKs (Hendricks-Taylor et al., 1997). Moreover, as shown in the SLP-76 deficient BMMCs, SLP-76 is necessary for the full activation of PLCγ1 and in turn for Ca^{2+} mobilization. Thus SLP-76 deficiency in BMMCs results in impairment of degranulation and IL-6 synthesis upon FcεRI cross linking (Pivniouk et al., 1999). To further assess the role of individual SLP-76 domains in mast cell signal transduction, BMMCs were retrovirally transduced with three different SLP-76 Y to F mutants. Detailed analysis showed that these tyrosine residues were dispensable for IL-6 secretion but not for degranulation and MAPK activation upon FcεRI stimulation. The most important tyrosine residue for FcεRI-mediated exocytosis appears to be Y145 (Kettner et al., 2003).

The most important member representing integral membrane adaptor proteins is LAT. LAT as a typical representative of TRAPs that are in general characterized by a short extracellular domain, transmembrane α-helix and a long cytoplasmic tail containing multiple tyrosine residues present within PTB consensus motifs. LAT was originally purified from the membrane fractions of activated Jurkat T cells (Zhang et al., 1998a). LAT functions as a central linker in T cells, as such tyrosine phosphorylated LAT was found to associate with Grb2, PLCγ1 (via SH2 domain) or indirectly with p85 subunit of PI3K and SLP-76. Mice with genetically disrupted LAT lack mature T lymphocytes in the periphery though they have normal B cells. Thymocytes in these mice are arrested at a double negative $CD8^-CD4^-$ stage. Thus LAT is required for proper T cell development (Zhang et al., 1999). LAT also plays a central role in the FcεRI-mediated signaling, however unlike in T cells it has no obvious role in mast cell development (Kimura et al., 2001; Saitoh et al., 2000). In T cells LAT is phosphorylated by Syk family kinase ZAP-70. Similarly, in mast cells LAT tyrosine phosphorylation is dependent on Syk. In LAT negative BMMCs tyrosine phosphorylation of both PLCγ isoforms and of SLP-76 is diminished. This in turn leads to impaired Ca^{2+} mobilization and diminution of mediators release upon IgE-initiated activation. FcεRI-dependent production of cytokines is either partially or completely inhibited. Moreover,

as determined using LAT negative mice, LAT appears to be crucial for in vivo histamine release too (Saitoh et al., 2000).

To further assess the role of LAT in mast cell signaling, LAT deficient BMMCs were reconstituted with LAT having one or more of the four membrane distal tyrosine (Y) residues mutated into phenylalanines (F). As revealed using these mutant cells, three membrane distal LAT tyrosines are responsible for Grb2 recruitment whereas Y 136 site binds PLCγ isoforms. The LAT-4YF mutants behave identically to LAT negative cells, while the other mutants provide partial reconstruction of LAT functions. Differential requirements for particular LAT tyrosine residues were established showing that the PLC binding site (Y136) is the most important one. LAT Y136 was determined to be essential for in vivo histamine release (Saitoh et al., 2003). Similarly, studies in mice homozygous for the Y136 LAT mutation show the importance of this tyrosine residue in PLCγ1 activation in T cells. Tyrosine phosphorylation of PLCγ1 was markedly reduced in these mutant T cells (Sommers et al., 2002). Since PLCγ1-dependent Ca^{2+} signaling pathway is important in setting the threshold for negative selection in the thymus (El-Hillal et al., 1997; Minoguchi et al., 1994), mutation in PLC-binding tyrosine in LATY136 knock-in mice resulted in lymphoproliferative disorder characterized by expansion of $CD4^+$ T cells producing T_H2 lymphokines. One of many consequences of this state is a very high level of circulating IgE (Saitoh et al., 2003). Most recently negative regulatory role of LAT has been proposed in mast cells (Malbec et al., 2004). Function of LAT was examined in two different types of mast cells: (1) BMMCs considered to be a mucosal type mast cells and (2) peritoneal cell derived mast cells (PCMCs), a novel type of cultured mast cells thought to represent serosal type mast cells. Both cell types were derived from knock-in mice expressing LAT with Y to F mutations of one (Y136), three (Y175, Y195, Y235) or all four (Y136, Y175, Y195, Y235) membrane distal tyrosines. Since the secretory response was enhanced in both types of mast cells bearing a mutation in the three most distal tyrosine residues, it was suggested that LAT plays a negative regulatory role in BMMCs as well as in PCMCs (Malbec et al., 2004).

1.4.6 Membrane rafts

Another aspect of cell signaling that should be taken into consideration is the participation of lipid/membrane rafts. These microdomains (enriched in sphingolipids and cholesterol) were originally identified by their resistance to Triton X-100 extraction (Brown and London, 1998; Simons and Ikonen, 1997), and are thought to be critical sites of signal propagation and membrane trafficking. LAT, alike some other transmembrane adaptors, contains a juxtamembrane CXXC palmitoylation motif, which is necessary for its partitioning into lipid rafts. As mentioned earlier, LAT association with GEMs seems to be essential for signaling through TCR, since the mutation of both cystein residues to alanines leads to decreased tyrosine phosphorylation of the linker molecule (Zhang et al., 1998b). Furthermore, LAT palmitoylation and its lipid raft targeting are required for LAT accumulation to the vicinity of activated TCR and for its ability to propagate TCR-initiated signaling events (Harder and Kuhn, 2000). Using LAT mutated in palmitoylation motif it was shown that LAT presence in lipid microdomains is also necessary for successful activation of Ras pathway and an increase in intracellular Ca^{2+} concentration upon TCR aggregation. The extracellular and transmembrane parts of LAT molecule are dispensable once the linker is anchored to GEMs (Lin et al., 1999). Nonetheless, the methods used for lipid raft detection, such as resistance to solubilization by the nonionic detergent Triton X-100 or sensitivity to cholesterol depletion, are indirect and difficult to interpret. Based on recent evidence from the biophysical and fluorescence imaging studies it has been proposed that lipid raft fraction may rather be a collection of separate heterogeneous membrane microdomains (Wilson et al., 2004).

In mast cells LAT was recovered from the light fraction containing detergent-resistant membranes after detergent extraction and subsequent sucrose density gradient centrifugation. Using such approach several other molecules including FcεRI and Lyn were isolated from the light density fraction, suggesting colocalization of those proteins also in the plasma membrane. However, studies based on high resolution electron microscopy and immunogold labeling of native membrane sheets reveal that LAT and FcεRI show little colocalization in resting cells and only transient in activated mast cells (Wilson et al., 2001). LAT molecules form small clusters in the non activated mast cell membranes and aggregate upon FcεRI-mediated activation into larger structures that associate at their margins with FcεRI clusters. At the periphery of FcεRI, LAT

could interact with γ subunit-bound Syk to become tyrosine phosphorylated by the enzyme. Because LAT and FcεRIs do not form mixed aggregates it has been suggested that distinct signaling domains are present on the plasma membrane. This seems quite feasible, since cross-linked FcεRI are internalized relatively rapidly via coated pits, whereas LAT is not. Thus LAT-organized signaling domains may be more stable then FcεRI-associated ones and provide more sustained signaling as the receptor level drops (Wilson et al., 2001). Most recently however the importance of LAT lipid raft localization for proper TCR-dependent signaling has been questioned; while using a fusion protein in which the extracellular part of LAT was replaced by corresponding part of non-raft transmembrane adaptor, linker of activation of X cells (LAX) (Zhu et al., 2005). LAX-LAT fusion protein was capable of restoring LAT functions such as MAPK activation and Ca^{2+} flux in LAT deficient cells, although the protein was not associated with lipid rafts. Moreover, in LAT-null mice expressing LAT-LAX fusion protein T cells development was fully rescued, indicating that LAT partitioning into lipid rafts might not be necessary for LAT function (Zhu et al., 2005).

1.4.7 Downstream signaling

Activation of early signaling molecules, such as Lyn, Fyn and Syk drives a complex network of intracellular events that regulate cellular functions. One of the key target molecules activated upon FcεRI aggregation are PLCγ isoforms, which cleave plasma membrane phosphatidylinositol 4, 5-bisphosphate in to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). PLCγ1 and PLCγ2 themselves become phosphorylated after recruitment to plasma membrane via association of pleckstrin homology (PH) domain with phosphatidylinositol 3,4,5-triphosphate (PIP3). Though the exact role of PIP3 in PLCγ activation is not fully defined and mainly mediated through the PLCγ-LAT interaction (Saitoh et al., 2000). Tyrosine phosphorylation of both PLCγ isoforms depends on Syk and on Tec family kinase Btk, Bruton's tyrosine kinase (Fluckiger et al., 1998; Sada et al., 2000). The two second messengers generated by active PLCγ, DAG and IP3 server to further propagate the FcεRI initiated signal. DAG activates several protein kinase C (PKC) isoforms and IP3 binds to its receptor on intracellular Ca^{2+} stores to release Ca^{2+} into the cytoplasm. Increased levels of intracellular Ca^{2+} lead to opening of plasma membrane store operated calcium channels that mediate sustained calcium signals (Turner and Kinet, 1999). Sustained cytoplasmic elevation of Ca^{2+} is required for

activation of transcription factor NFAT (nuclear factor of activated T cells), which functions to activate transcription of cytokine genes as well as for activation of family of serine/threonine PKCs, key molecules involved in mast cell exocytosis (Blank and Rivera, 2004; Turner and Kinet, 1999). Lyn and LAT seem dispensable for Gab2 phosphorylation, which instead relies presumably on Fyn activity leading to subsequent activation of PI3K. Accordingly, PI3K-mediated activation is defective in BMMCs derived from mice lacking Gab2. Moreover allergic reactions such as passive cutaneous and systemic anaphylaxis are markedly impaired in Gab2 deficient mice (Gu et al., 2001). Nonetheless, in contrast to LAT-negative mast cells only a little reduction in calcium response was observed in Gab-null mast cells. Apparently both LAT and Gab2 dependent pathways complement at the level of Ca^{2+} mobilization and PKC activation (Blank and Rivera, 2004). This assumption seems to be further supported by the observation that in BMMCs derived from mice with defect in p85α or p85β regulatory subunit of PI3K tyrosine, phosphorylation of PLCγ1 and IP3 production remain unaffected. Moreover the inhibitor of PI3K, wortmannin, had no effect on phosphorylation of PLCγ1 and subsequent IP3 production in $CD34^{+}$ peripheral blood progenitor derived human mast cells, although high concentrations of wortmannin partially inhibited degranulation of these cells (Tkaczyk et al., 2003).

Nonetheless as documented most recently, mast cells defective in p110δ isoform of PI3K, a catalytic subunit of class IA PI3K primarily expressed in leukocytes show impaired SCF-mediated in vitro proliferation, adhesion and migration along with diminished antigen-IgE initiated degranulation and cytokine production. Moreover, compared to wild type, the number of mast cells were significantly lowered in mice expressing $p110\delta^{D910A}$, a loss of function allele, though the reduction in distinct mast cells populations differ in various anatomical locations. In contrast no significant differences were found in mast cell counts in back dermis and mucosa of the stomach. Also the passive cutaneous anaphylaxis in $p110\delta^{D910A/D910A}$ mice was markedly reduced suggesting that p110δ has an important role in mast cell homeostasis and in the allergic responses (Ali et al., 2004). In addition microinjection of antibodies raised against p110β and P110δ PI3K subunits into RBL-2H3 cells led to the delayed Ca^{2+} release from intracellular stores as well as to diminution of sustained phase of Ca^{2+} response. Since the lipid product of PI3K activity, PIP3, is able to promote predominantly the enzymatic activity of already tyrosine-

phosphorylated PLCγ, it has been proposed that impaired Ca^{2+} response is presumably a consequence of reduced activation of both PLCγ isoforms (Smith et al., 2001).

Also Vav1 protein which is primarily recognized as guanine nucleotide exchange factor with a preference for Rac GTPases was established to significantly participate in PLCγ mediated calcium signaling. In Vav1 knockout mast cells the activation of both PLCγ isoforms is inhibited, although their association with the scaffolding protein LAT is only modestly diminished upon FcεRI aggregation. In addition phosphorylation of LAT and SLP-76 along with its targeting to LAT in IgE-activated Vav1-deficient BMMCs remains unaffected. Thus the defect in calcium response in LAT-, SLP-76- and Vav1-deficient cells might be a result of a failure to recruit active Vav1 to the LAT organized-signaling complex (Manetz et al., 2001).

Lack of activity of lipid phosphatase SHIP, an SH2-containing inositol polyphosphate 5-phosphatase, has been proposed to account for enhanced degranulation in Lyn-deficient mast cells. An increased level of PI3K generated product, PIP3, in Lyn-negative BMMCs seems to be a consequence of diminished phosphatase activity of SHIP rather than of higher PI3K activity (Hernandez-Hansen et al., 2004). SHIP itself is responsible for a cleavage of membrane-associated PIP3 to yield phosphatidylinositol 3,4 bisphosphate (PIP2) and thereby reduces targeting of certain PH domain containing proteins to the plasma membrane and their activation. Using SHIP-negative BMMCs its was shown that SHIP decreases IL-6 mRNA and in turn protein production by inhibiting NF-κB transcription factor mainly in Akt-dependent manner (Kalesnikoff et al., 2002). Moreover as demonstrated earlier SHIP plays an important role in mast cell degranulation by setting the threshold for and limiting the exocytosis process (Huber et al., 1998). This finding appears to be in line with the most recent observation that SHIP is critical in the down regulation of mast cell degranulation at supraoptimal antigen levels. Indeed the overall Ca^{2+} flux in SHIP-deficient BMMCs is by far higher at optimal as well as at supraoptimal antigen concentrations compared with wild type BMMCs (Gimborn et al., 2005a). In addition, SHIP may be recruited to the plasma membrane via FcεRI-β chain; at the supraoptimal concentrations of antigen β subunit associate preferentially with positive regulators of mast cell activation (Gimborn et al., 2005b).

Another phosphatase known to participate in immune receptor signaling is SH2 domain containing phosphatase (SHP)-1. SHP-1 along with SHP-2 belongs to the family of non receptor tyrosine phosphatases and both are expressed in mast cells. To assess the role of SHP-1 in mast cell signaling wild type or phosphatase-inactive form of SHP-1 were overexpressed in RBL-2H3 cells. In such cells FcεRI-mediated phosphorylation of β and γ receptor subunits was decreased, whereas, overexpression of dominant negative form of SHP-1 led to enhancement of phosphorylation of the receptor subunits. Despite SHP-1 has a negative regulatory effect on Syk tyrosine phosphorylation, it positively modulates phosphorylation of JNK, c-Jun N-terminal kinase, resulting in increased TNF-α synthesis (Xie et al., 2000).

1.5 Exocytosis

Exocytosis is a process in which a membrane bound intracellular vesicle moves to the plasma membrane and subsequently fuses with plasma membrane. Many cellular processes involve exocytosis. For example a few of the processes that use exocytosis are: (1) secretion of proteins like enzymes, peptide hormones and antibodies from cells; (2) turnover of plasma membranes; (3) release of neurotransmitters from presynaptic neurons; (4) placement of integral membrane proteins; (5) acrosome reaction during fertilization; (6) antigen presentation during the immune response; (7) recycling of plasma membrane bound receptors; (8) cell membrane repair.

Secretion of inflammatory mediators by exocytosis in mast cells has been studied extensively. In most cases exocytosis markers used were histamine or β-glucuronidase. But many of the processes that involve exocytosis as listed above are yet to be addressed in mast cells. Out of all listed, cell membrane repair turns out to be an important aspect because of its involvement in recently postulated autoimmunity and danger hypothesis (Andrews, 2005). Inhibitors of exocytosis play a crucial role in elucidating the involvement of exocytosis in cell membrane repair. Even though many potent inhibitors have been screened, vacuolin-1 (Fig.2), a triazine based molecule inhibiting Ca^{2+}-mediated exocytosis, grabbed the attention of critics (Cerny et al., 2004; Huynh and Andrews, 2005).

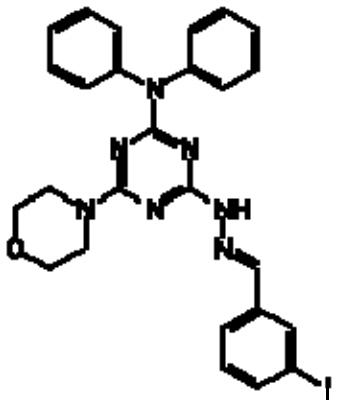

Fig 2. Molecular structure of vacuolin-1.

Exocytosis and endocytosis are important cellular functions of outward and inward vesicular transport involved in intra- and inter-cellular communications. They are accomplished through the release and uptake of chemical signals important in a variety of cellular functions, such as neurotransmitter release and receptor internalization. Ca^{2+}-dependent exocytosis and endocytosis have also been implicated in the repair of injured plasma membrane (Reddy et al., 2001; Idone et al., 2008). Using a small chemical vacuolin-1, which induces formation of large swollen structures derived from lysosomes and endosomes, Cerny et al. attempted to determine the role of lysosome exocytosis in membrane repair. Pretreatment of human HeLa cells with vacuolin-1 inhibited Ca^{2+}-ionophore induced lysosome exocytosis but not repair of damaged plasma membrane, suggesting that lysosomes are dispensable for membrane resealing (Cerny et al., 2004). Further studies performed under slightly different conditions however showed normal exocytosis and membrane resealing in vacuolin-1-pretreated HeLa and NRK cells, despite formation of the large swollen structures. These data suggested that lysosomes cannot be excluded as possible agents of membrane repair in vacuolin-1-treated cells (Huynh and Andrews, 2005). In attempts to solve these discrepancies, other experiments showed that vacuolin-1 inhibited exocytosis only if cells were activated with ionomycin in solutions supplemented with bovine serum albumin (BSA) or serum, but not in protein-free solutions (Steinhardt, 2005). Because ionomycin is not physiological Ca^{2+} inducer and has some serious side effects, including a decline in ATP content of the cells (Gmitter et al., 1996) further studies are required to elucidate the role of exocytosis in membrane resealing in professional secretory mast cells.

2. AIMS

Long-range goal of the laboratory where this thesis was worked-out has been the understanding of molecular mechanisms involved in mast cell activation leading to exocytosis of preformed mediators and production of cytokines. Recent studies have been directed mostly towards the structure-function analyses of transmembrane and cytoplasmic adaptor proteins and correlation between exocytosis induced by aggregation of the FcεRI and other plasma membrane components and early signaling pathways. This thesis is mainly focused on deeper understanding of the role of NTAL adaptor protein and changes in the plasma membrane in the course of mast cells activation. Some methodological tasks were also included. Specific aims of this thesis were:

2.1 **To contribute to elucidating the role of NTAL and Grb2 in mast cell signaling.** RBL-2H3 cells were used as a model system to achieve the following sub-aims:

2.1.1 Production of vectors for knock-down of NTAL and Grb2.

2.1.2 Isolation of RBL-2H3-derived cell lines with decreased amount of NTAL and/or Grb2.

2.1.3 Screening of RBL-2H3-derived clones for NTAL and/or Grb2 expression by immunoblotting procedures and isolating stable cell lines.

2.1.4 To analyze β-glucuronidase secretion in cells deficient in expression of NTAL and/or Grb2.

2.1.5 To analyze Ca^{2+} mobilization in cells deficient in the expression of NTAL and/or Grb2.

2.1.6 To compare tyrosine phosphorylation of proteins in activated NTAL-deficient and control cells.

2.1.7 To determine the effect of reduced expression of NTAL on the expression of surface FcεRI.

2.2 **To elucidate relationship between mast cell exocytosis and membrane resealing**. Small chemical vacuolin-1 and various pharmacological inhibitors, and RBL-2H3 cells and BMMCs were used as model systems to achieve the following sub-aims:

2.2.1 To determine the effect of vacuolin-1 treatment on morphology of RBL-2H3 cells and BMMCs.

2.2.2 To determine the effect of vacuolin-1 treatment on exocytosis in RBL-2H3 cells and BMMCs activated via antigen, ionophore or thapsigargin.

2.2.3 To determine the effect of various pharmacological inhibitors on vacuolin-1-induced changes in RBL-2H3 cells and BMMCs.

2.2.4 To determine the effect of vacuolin-1 treatment on expression of FcεRI.

2.2.5 To determine the effect of vacuolin-1 treatment on cell membrane resealing after SLO-mediated damage.

2.3 **To improve performance of PCR by modifying composition of PCR mixes.** Genomic DNA, plasmid DNA and cDNA were used as templates for achieving the following methodological sub-aims:

2.3.1 Identification of additives enhancing amplification efficiency and/or reducing non-specific amplification.

2.3.2 Use of new amplification strategies for identification of changes in gene expression and for determination of genotypes.

3. SUMMARY OF METHODS

3.1 To prepare NTAL and or Grb2 knock-downs, vector-mediated RNAi strategy was used. Target oligonucleotides were cloned into mU6pro vector and introduced into the cells by electroporation along with pCiNeo vector as selection marker in 1:9 ratio.

3.2 Geneticin-resistant clones were isolated and lysates were prepared and checked for expression levels of the target proteins by immunoblotting.

3.3 Phosphorylation of respective proteins was analyzed by developing the blots with phospho-specific antibodies.

3.4 Selected clones were subjected to functional analysis by checking β-glucuronidase release after activation with antigen. Free intracellular Ca^{2+} was measured by labeling the cells with Fura2 before and after activation and read by spectrophotometry.

3.5 RBL-2H3 cells and BMMCs were pretreated with vacuolin-1 and morphological changes were observed after staining smears of the cells with Giemsa-Romanowski stain. Cell viability was checked by trypan blue staining. Cells pretreated with vacuolin-1 or not were subjected to activation via antigen, ionophore or thapsigargin. Exocytosis was checked by measuring β-glucuronidase secretion. Pharmacological inhibitors were selected based on inhibition of vacuolin-1-induced morphological changes.

3.6 Cell membrane repair assay employed streptolysin O to make holes into plasma membrane. Membrane damage was evaluated after staining the cells with propidium iodide and flow cytometry analysis.

3.7 RNA was isolated from RBL-2H3 cells and or cholestatic rat liver tissue. cDNA was prepared and subjected to qRT-PCR with SYBR green.

3.8 Tetraalkylammonium derivatives were screened as additives for enhancement of amplification efficiency and reduction of non-specific amplification and formation of primer dimmers in qRT-PCR employing SYBR green.

All methods were described in detail in relevant publications attached under results section. Here only production of siRNA constructs and other relevant methods are described in detail.

Procedure used for cloning hairpin siRNA oligonucleotides into the mU6pro vector

Resuspend DNA oligonucleotides at 0.25 nmoles/µl (water or TE). Oligonucleotides do not need to be phosphorylated or purified.

Annealing of oligonucleotides:

20 µl oligo1 (=100 nmoles/ml final concentration)
20 µl oligo2 (=100 nmoles/ml final concentration)
5 µl water
5 µl 10X annealing buffer (10X = 1 M NaCl, 100 mM Tris-HCl, pH 7.4)
50 µl total (= 5 nmoles total of oligo)
Note: the annealing volume will need to be scaled down for 10 nmol scale oligonucleotide synthesis, as there will not be sufficient oligo for the above.
Heat 1,5 ml tubes with water to 85-95^{o}C in microwave, immediately add oligos and allow to cool to room temperature (usually an hour or two; overnight is fine). Alternately, a PCR machine can be used for annealing. Store the annealed template at –20^{o}C.

Ligation:

After annealed template has cooled, dilute some of it 1:4000 in 0.5X annealing buffer. Use 1 µl of the 1:4000 dilution for ligation to 30-50 ng of the Bbs1/Xba1restriction digested/gel purified mU6pro vector in a 10 µl volume. With concentrated ligase, this reaction can be performed for 10-30 min at room temperature.

Colony screening:

Cloning efficiency is usually high enough that one or several random colonies can be chosen for sequencing without screening. If necessary, it is possible to use either the mU6F2 or f1F forward primers in combination with the M13R2 reverse primer (or a similar M13 reverse primer) for colony PCR screening. Expect about a 650 bp band if correct, 1350 pb band for parental vector

note that larger = recircularized vector and smaller = correct product, since the eGFP gene is deleted during the cloning with Bbs1 and Xba1).

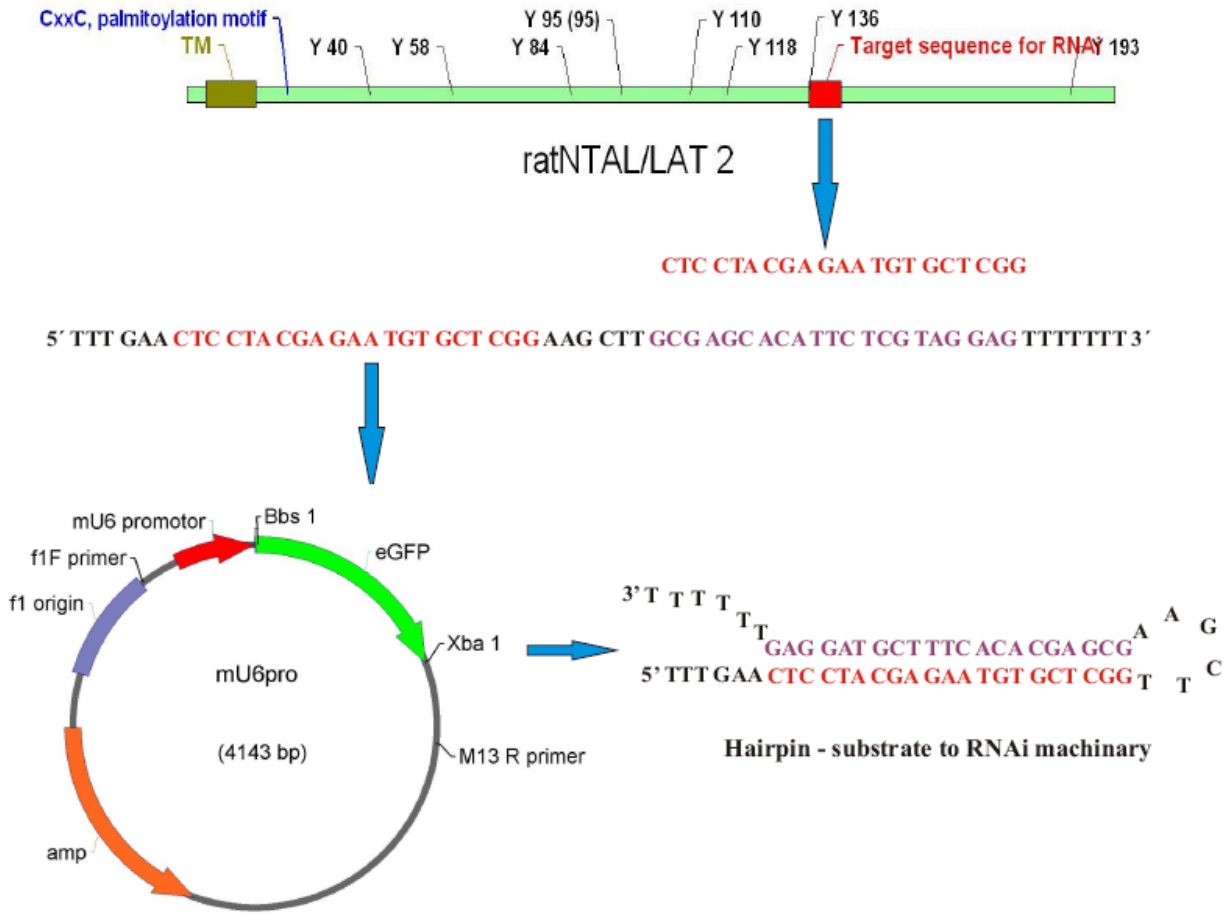

Fig. 3. Schematic diagram of NTAL protein and target region for RNAi; also depicted are NTAL target oligos, cloning strategy, and properties of hairpin which is produced and is involved in RNAi.

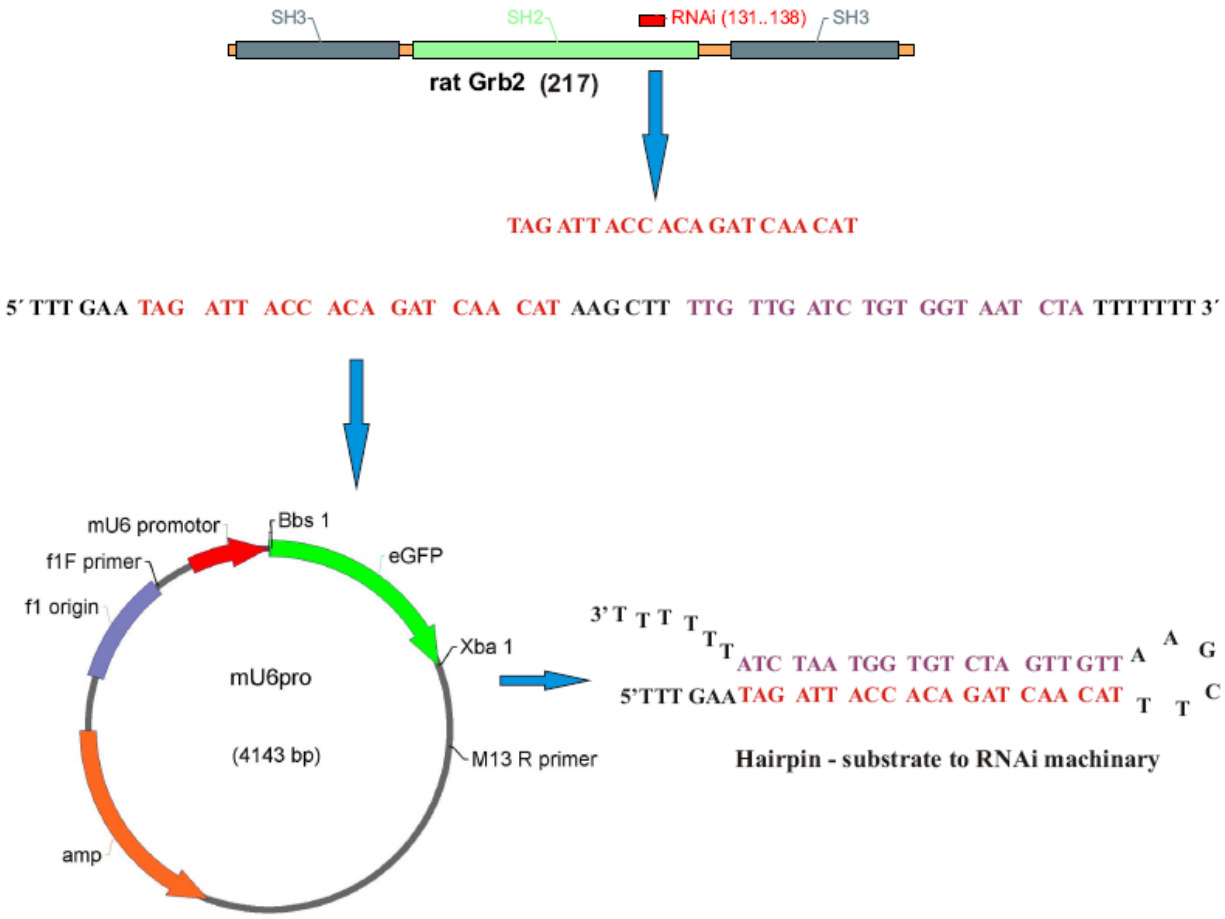

Fig. 4. Schematic diagram of Grb2 protein and target region for RNAi; also depicted are Grb2 target oligos, cloning strategy, and properties of hairpin which is produced and is involved in RNAi.

4. RESULTS

4.1 Regulation of Ca^{2+} signaling in mast cells by tyrosine-phosphorylated and unphosphorylated non-T cell activation linker, NTAL

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The aim of this study was to contribute to elucidating the role of NTAL adaptor protein in mast cell signaling. Major tasks were to prepare RBL-2H3 derived stable cell lines with enhanced or decreased expression of NTAL and decreased expression of Grb2, analyze them in various functional and immunochemical assays and compare them with wild-type cells. I have contributed to this work by production and characterization of various DNA vectors used for production of cell lines with reduced levels of NTAL and Grb2, performing isolation of knock-downs and controls and initial characterization of them, mainly by immunoblotting examination of the proteins of interest, analysis of their ability to release β-glucuronidase and calcium mobilization in antigen- or thapsigargin-activated cells. I was involved at all stages of this work, including outline of the strategies used for production of genetically modified cell lines, evaluation of the experiments and writing the corresponding paragraphs. This is in part reflected by me to be the second author of this study of 8 authors.

4.2 Vacuolin-1-modulated exocytosis and cell resealing depend on cell origin and mode of activation.

Submitted to EMBO reports

The aim of this study was to contribute to the understanding the relationship between exocytosis and membrane resealing. Control and vacuolin-1-treated RBL-2H3 or BMMCs cells were used. Major tasks were to study the properties of cells after vacuolin-1 treatment: morphology, exocytosis, changes in signaling pathways, screening of selected pharmacological inhibitors and able to inhibit, analysis of membrane resealing after streptolysin O treatment. Being the first author of this paper, I participated in the conceptual outline of the study, did all experimental work, except tyrosine phosphorylation studies, analysed data obtained and participated in writing the manuscript.

Vacuolin-1-modulated exocytosis and cell resealing depend on cell origin and mode of activation

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Running title: Vacuolin-1, exocytosis and membrane repair

Key words: exocytosis, plasma membrane, vacuolin-1, mast cell

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ABSTRACT

The role of lysosomal exocytosis in repair of damaged plasma membrane is incompletely understood. A small chemical vacuolin-1 was shown to inhibit Ca^{2+} ionophore-induced exocytosis depending on experimental conditions used, and led to ambiguous conclusions on the role of lysosomal exocytosis in membrane repair. Here we show that rat basophilic leukemia (RBL-2H3) cells treated with vacuolin-1 and activated through the high-affinity IgE receptor exhibited enhanced exocytosis. Under identical conditions of activation, exocytosis was inhibited in bone marrow-derived mast cells (BMMCs). Thapsigargin- and Ca^{2+} ionophore A23187-induced exocytosis also showed different sensitivity to inhibitory effect of vacuolin-1. If the cells were permeabilized with bacterial toxin streptolysin O (SLO), vacuolin-1 enhanced the resealing in RBL-2H3 cells but inhibited it in BMMCs. Our data support the concept that lysosomal exocytosis is involved in membrane repair.

INTRODUCTION

Exocytosis and endocytosis are important cellular functions of outward and inward vesicular transport involved in intra- and inter-cellular communications. They are accomplished through the release and uptake of chemical signals important in a variety of cellular functions, such as neurotransmitter release and receptor internalization. Ca^{2+}-dependent exocytosis and endocytosis have also been implicated in the repair of injured plasma membrane (Reddy et al., 2001; Idone et al., 2008). Using a small chemical vacuolin-1, which induces formation of large swollen structures derived from lysosomes and endosomes, Cerny et al. attempted to determine the role of lysosome exocytosis in membrane repair. Pretreatment of human HeLa cells with vacuolin-1 inhibited Ca^{2+}-ionophore induced lysosome exocytosis but not repair of damaged plasma membrane, suggesting that lysosomes are dispensable for membrane resealing (Cerny *et al.*, 2004). Further studies performed under slightly different conditions however showed normal exocytosis and membrane resealing in vacuolin-1-pretreated HeLa and NRK cells, despite formation of the large swollen structures. These data suggested that lysosomes cannot be excluded as possible agents of membrane repair in vacuolin-1-treated cells (Huynh and Andrews, 2005). In attempts to solve these discrepancies, other experiments showed that vacuolin-1 inhibited exocytosis only if cells were activated with ionomycin in solutions supplemented with bovine serum albumin (BSA) or serum, but not in protein-free solutions (Steinhardt, 2005). Because ionomycin is not physiological Ca^{2+} inducer and has some serious side effects, including a decline in ATP content of the cells (Gmitter *et al.*, 1996), we decided to examine the effect of vacuolin-1 on exocytosis in specialized secretory cells activated by physiological and nonphysiological activators. We used rat basophilic leukemia (RBL-2H3) cells and mouse bone marrow-derived mast cells (BMMCs) pretreated with vacuolin-1 and activated through the high-affinity IgE receptor (FcεRI), or by Ca^{2+} ionophore A23187 or thapsigargin, an agent that induces the release of Ca^{2+} from intracellular stores by inhibiting endoplasmic reticulum ATPase (Thastrup *et al.*, 1989). We also analyzed the effect of various pharmacological inhibitors on formation of vacuoles in vacuolin-1-treated cells, as well as the effect of vacuolin-1 on early stages of FcεRI signaling and on membrane resealing in cells permeabilized with bacterial toxin streptolysin O (SLO). Our data indicate that the inhibitory effect of vacuolin-1 on exocytosis and repair of injured plasma membrane depends not only on conditions of activation but also on cell

origin. For the first time the study supports the notion that lysosomal exocytosis is involved in membrane repair even in professional secretory cells.

RESULTS AND DISCUSSION

Vacuolin-1-induced changes in mast cells morphology

In pilot experiments we determined the effect of vacuolin-1 on mast cell morphology. Incubation of RBL-2H3 cells or BMMCs with 10 μM vacuolin-1 in complete culture media for 3 h resulted in appearance of numerous vacuoles in cytosolic compartment of the cells (Fig 1A-D). Size of the vacuolin-1-treated cells rose (Fig 1E and F) as reflected by mean increase in diameter of RBL-2H3 cells from 14.8 ± 0.2 μm (mean ± S.D., n = 3) to 18.8 ± 0.3 μm, and BMMCs from 14.6 ± 0.2 μm to 17.3 ± 0.2 μm.

To decide whether formation of vacuoles depends on specific metabolic pathways we examined cells exposed to various concentrations of pharmacological inhibitors and 10 μM vacuolin-1. Data presented in Table 1 show that most of the drugs tested, including Cl^- and/or K^+ channel blockers [indanyloxyacetic acid 94 (IAA-94), (dihydroindenyl)oxy/alkanoic acid (DIOA), 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS) and glybenclamide], protein kinase C (PKC) inhibitor (tamoxifen), phosphoinositid-3 kinase (PI3K) inhibitor (wortmannin), Src and Syk family kinase inhibitors (PP2 and piceatannol) and non-muscle myosin (NMM) II ATPase activity inhibitor (blebbistatin) had no effect on vacuolin-induced formation of vacuoles even at such high doses tested that were often toxic. The only exception was macrolide antibiotic bafilomycin A1, a potent inhibitor of vacuolar H^+-ATPase (Bowman *et al.*, 1988). Treatment with 0.01 μM bafilomycin completely inhibited vacuolin-1-induced formation of vacuoles in both RBL-2H3 cells and BMMCs without any decrease in cell viability, as confirmed by trypan blue staining (not shown); toxic effects were only observed at >50-fold higher concentrations of bafilomycin. These data indicate that vacuolar H^+-ATPase is involved in vacuolin-1-induced formation of vacuoles. In its sensitivity to bafilomycin A1, vacuolin-1 resembles the Vac A toxin produced by Helicobacter pylori, which mediates an influx of anions into endosomes leading, in turn, to increased activity of vacuolar H+-ATPase, osmotic swelling and formation of vacuoles (Papini et al., 1993; Cover and Blanke, 2005). Vacuolin-1 could thus also enhance the influx of anions into lysosomes and endosomes resulting in their osmotic swelling. Inhibition of vacuole production was also observed in cells pretreated with nonlethal doses of myosin light chain (MLC) kinase inhibitors ML-7 and ML-9.

These two inhibitors were effective only in BMMCs, suggesting that MLC kinase could be responsible at least in part for the observed differences in vacuolin-1 sensitivity between RBL-2H3 cells and BMMCs (see below).

Sensitivity of exocytosis to vacuolin-1

To check whether vacuolin-1 inhibits secretory response in mast cells, RBL-2H3 and BMMCs were simultaneously sensitized with IgE and treated with 10 μM vacuolin-1. After 3 h incubation unbound IgE and vacuolin-1 were washed out, cells were activated with antigen in the presence of freshly added vacuolin-1, and the amount of β-glucuronidase released was determined. Control cells were sensitized and activated in the same way except that vacuolin-1 was replaced by vehicle [(dimethylsulfoxide (DMSO)], which at concentrations used (<1%) had no effect on secretory response (not shown). Fig 2A shows that antigen (TNP-BSA)-induced secretory response was more profound in BMMCs than in RBL-2H3 cells at both time intervals, 5 and 30 min. Vacuolin-1 enhanced slightly, but significantly, the FcεRI-mediated secretory response in RBL-2H3 cells. Unexpectedly, activation of vacuolin-1 treated BMMCs under the same conditions completely inhibited the secretory response. This inhibition was not due to a decreased amount of FcεRI on the surface of the cells determined by flow cytometry analysis (not shown). Furthermore, the cells did not bind annexin-V-fluorescein isothiocyanate (FITC) conjugate (not shown), indicating that their membranes remained intact (Smrz *et al.*, 2008). Importantly, vacuolin-1 also induced inhibition of exocytosis in BMMCs activated in BSA-free buffered salt solution (BSS). This implies that the molecular mechanism of inhibition is more complex then expected (Steinhardt, 2005). To check whether the observed inhibitory effect was confined to FcεRI-mediated activation, we also examined the secretory response in cells activated by Ca^{2+} ionophore A23187 or thapsigargin. Data in Fig 2B indicate that vacuolin-1 inhibited A23187-induced secretion both in BMMCs and in RBL-2H3-cells. This inhibitory effect could be related to known multiple effects of A23187 on plasma membrane, such as decreased membrane packing measured by MC540 binding (Smrz *et al.*, 2007), or increased plasmalemmal Ca^{2+} ATPase activity resulting in a decline in ATP content of the cells (Gmitter *et al.*, 1996). It should be noted that this inhibition was independent of the presence of BSA in BSS (not shown). Interestingly, when vacuolin-1-treated cells were activated by endoplasmic reticulum ATPase inhibitor, thapsigargin, (Thastrup *et al.*, 1989), secretory response was slightly

enhanced in RBL-2H3 (significantly at 5 min after triggering) but decreased only insignificantly in BMMCs (Fig 2C). To find out whether the observed effect of vacuolin-1 on antigen-activated cells was confined just to certain time intervals or concentrations of vacuolin, time- and dose-responses were studied. Pretreatment of RBL-2H3 cells for 15 h with 10 μM vacuolin-1 had no significant effect on spontaneous release of β-glucuronidase (Fig 2D). Enhanced secretory response in antigen-activated cells was observed at all time intervals of vacuolin-1 treatment, peaking at 12 h. In BMMCs, secretory response was inhibited in cells treated for 3 - 15 h, confirming that these cells are extremely sensitive to 10 μM vacuolin-1. To construct the dose-response curves, cells were first incubated with vacuolin-1 for 3 h and then activated with antigen. Pretreatment with various concentrations of vacuolin-1 (from 0.5 to 90 μM) enhanced in a dose-dependent manner the FcεRI-induced secretory response in RBL-2H3 cells but decreased it in BMMCs (Fig 2E). These data suggest that FcεRI-signaling pathways might be affected by vacuolin-1 in different ways, depending on cell type studied.

Vacuolin-1 and tyrosine phosphorylation in BMMCs

The earliest biochemically defined step in FcεRI triggering is tyrosine phosphorylation of FcεRI β and γ subunits, followed by phosphorylation of other substrates, such as linker for activation of T cells (LAT), non-T cell activation linker (NTAL), Akt and MAP kinase Erk. In further experiments we therefore tested whether the observed inhibition of exocytosis in vacuolin-1-treated and antigen-activated BMMCs could reflect an inhibition of those early signaling events. Fig 3A, documents strong tyrosine phosphorylation of FcεRI β and γ subnits in FcεRI-activated cells pretreated with vehicle alone and almost the same response in vacuolin-1-pretreated cells. Similarly, tyrosine phosphorylations of LAT and NTAL (Fig 3B), Akt (Fig 3C) and Erk (Fig 3D) were comparable in vehicle- or vacuolin-1-treated cells. All these data indicate that early receptor-mediated tyrosine phosphorylations are not affected by vacuolin-1 and are not responsible for the observed inhibitory effect of vacuolin-1 on antigen-induced exocytosis in BMMCs.

Vacuolin-1 and Ca^{2+} uptake in BMMCs

Early FcεRI-triggered activation events are followed by release of Ca^{2+} from intracellular stores, and subsequent enhanced uptake of extracellular Ca^{2+}. Because these changes are essential for exocytosis, we next examined the uptake of extracellular Ca^{2+} in control and vacuolin-1-treated cells. When IgE-sensitized RBL-2H3 cells or BMMCs were activated by antigen, the enhanced uptake of $^{45}Ca^{2+}$ was comparable in both vehicle- and vacuolin-1-treated cells (Fig 4A). For comparison we also determined $^{45}Ca^{2+}$ uptake in cells activated by A23187 and again, the uptake was comparable in both control and vacuolin-treated cells (Fig 4). A small yet significant inhibition of $Ca2^{+}$ uptake was only observed in vacuolin-pretreated and thapsigargin-activated BMMCs (Fig 4C), which could explain some decrease in exocytosis in cells activated by thapsigargin (Fig 2C). The combined data indicate that signaling pathways from FcεRI to Ca^{2+} uptake are not affected by vacuolin-1 in FcεRI-activated BMMCs.

Effect of vacuolin-1 on filamentous (F) actin formation

Translocation of secretory granules and their exocytosis in mast cells is negatively regulated by F- actin (Frigeri and Apgar, 1999; Tolarová et al., 2004). We therefore tested the effect of vacuolin-1 on F-actin formation in activated cells. Our data show that the amount of F-actin in antigen-activated RBL-2H3 cells is enhanced and that vacuolin-1 significantly inhibits this rising at both time intervals chosen, 2 and 8 min (Fig 5). The observed inhibition of F-actin polymerization could explain enhanced exocytosis in vacuolin-1 treated cells. No increase, but rather decrease, in F-actin polymerization was observed in activated BMMCs and no evidence of the effect of vacuolin-1 on F-actin polymerization was obtained. Different properties of F-actin in activated RBL-2H3 cells and BMMCs and the observed differences in sensitivity of antigen-mediated exocytosis to vacuolin-1 could be related to the fact that RBL-2H3 cells are adherent and BMMCs grow in suspension. To address this issue, we used RBL-2H3 cells grown in suspension for 24 h and found that FcεRI-induced exocytosis is also enhanced in this case by vacuolin-1 (not shown). These data indicate that differences in adhesion properties of RBL-2H3 and BMMCs are not responsible for the observed differences between these two cell types.

Effect of vacuolin-1 on membrane repair

Recent experiments with bacterial toxin SLO showed that membrane resealing and removal of SLO-containing pores requires Ca^{2+}-dependent endocytosis (Idone *et al.*, 2008). To determine whether vacuolin-1-sensitive structures are involved in membrane resealing we compared repair of SLO-permeabilized plasma membranes in control and vacuolin-1-treated cells. When RBL-2H3 cells were permeabilized with SLO an increase in the number of propidium iodide (PI)-positive cells was observed with rising concentrations of SLO (Fig 6A, $+Ca^{2+}$ and summary data in C). Pretreatment with 10 μM vacuolin-1 resulted in a significant decrease in the count of PI-positive cells at all concentrations of SLO examined, which is in accord with observed enhanced exocytosis in vacuolin-1-treated RBL-2H3 cells (Fig 2A and D). When experiments were repeated in calcium-free solutions, more RBL-2H3 cells were PI positive with the same concentrations of SLO, and vacuolin had no significant effect on PI staining (Fig 6A, $-Ca^{2-}$ and summary data in C). These findings support previous data showing that removal of SLO-containing membrane pores is Ca^{2+} dependent (Walev et al., 2001; Idone et al., 2008) and complement them by showing that vacuolin-1 promotes this process. BMMCs showed higher resistance to SLO and, importantly, treatment with vacuolin-1 did not enhance removal of membrane pores, but rather reduced it (reflected in higher percentage of PI-positive cells; the increase was significant at 3 U SLO/ml). As expected, the number of PI-positive BMMCs was enhanced in the absence of $Ca^{2+}$and again vacuolin had no effect on this parameter. Thus vacuolin-1-induced inhibition of FcεRI exocytosis in BMMCs has its counterpart in the inability of vacuolin-1 to promote membrane repair after SLO permeabilization. Correlation between vacuolin-1-modulated exocytosis and membrane repair in SLO-permeabilized cells suggest a coupling of lysosomal exocytosis and endocytosis (compensatory endocytosis) in mast cells.

CONCLUSIONS

Data presented in this study show unexpected differences in sensitivity of FcεRI-induced exocytosis to vacuolin-1 in two mast cell types: exocytosis was enhanced in RBL-2H3 cells and inhibited in BMMCs. The molecular basis of this divergence is unknown but could be related to the observed differences in vacuolin-1 sensitivity of antigen-mediated actin polymerization and action of some drugs. Our finding that sensitivity of exocytosis to vacuolin-1 correlates with membrane repair after treatment of the cells with bacterial toxin SLO supports the concept that lysosomal exocytosis and compensatory endocytosis are involved in membrane repair even in professional secretory cells.

METHODS

Experiments were performed with RBL-2H3 cells, clone2H3, and mouse BMMCs derived from C57BL/6J mice.

Statistical analysis. Means ± S.D.were calculated from at least 3 independent experiments. Statistical significance of differences were analyzed using Student's t-test, except data in Fig. 5A, which were evaluated by Wilcoxon signed-rank test (http://faculty.vassar.edu/lowry/wilcoxon.html). A difference with p-value <0.05 was considered significant.

For detailed description of materials and methods, see supplementary information online.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Figure legends

Fig 1 | Morphology of vacuolin-1-treated RBL-2H3 cells and BMMCs. (A-D) Cells were incubated in complete culture media with vehicle (0.1% DMSO) alone (A, B) or with 10 μM vacuolin-1 (C and D). After 3 h the cells were smeared and stained with May-Grünwald and Giemsa-Romanowski procedure. Bar, 10 μm. (E, F) RBL-2H3 cells (E) or BMMCs (F) were treated with vehicle (black line) or 10 μM vacuolin-1 (red line) as above. Size of the cells in suspension was determined by means of Vi-cell XR cell viability analyzer.

Fig 2 | Different effect of vacuolin-1 on exocytosis in RBL-2H3 cells and BMMCs. (A) Cells were sensitized with TNP-specific IgE and concurrently exposed to vehicle (black columns) or 10 μM vacuolin-1 (red columns). After 3 h the cells were washed and activated in the presence of vehicle or vacuolin with antigen (TNP-BSA; 250 ng/ml). The amount of β-glucuronidase released into supernatant was determined at different time intervals after triggering. (B, C) Cells were treated with vehicle or vacuolin-1 as in A, then exposed to Ca^{2+} ionophore A23187 (B; 1 μM) or thapsigargin (C; 2 μM) and analyzed as in A. (D) Cells were exposed for various time intervals to 10 μM vacuolin-1 and concurrently sensitized for the last 3 h with TNP-specific IgE. The cells were activated with TNP-BSA (250 ng/ml) and β-glucuronidase released was determined 15 min after triggering. (E) Cells were exposed for 3 h to different concentrations of vacuolin-1 and simultaneously sensitized with TNP-specific IgE followed by processing as in D. The data are presented as means ± SD calculated from 3-4 experiments. Asterisks indicate significant differences.

Fig 3 | Vacuolin-1 does not inhibit early signaling events in FcεRI-activated BMMCs. Cells were incubated with vehicle (-Vacuolin-1) or 10 μM vacuolin (+Vacuolin-1) and concurrently sensitized with IgE. After 3 h the cells were washed and activated for 2 or 5 min with TNP-BSA prior to lysis in buffer containg 0.2% Triton X-100 (A) or 1% NP-40 and 1% n-dodecyl β-d-maltoside (B - D). IgE-FcεRI complexes in postnuclear supernatants were immunoprecipitated (IP) with anti-IgE-specific antibodies and the immunocomplexes were fractionated by SDS-PAGE and analyzed by immunoblotting (IB) with phosphotyrosine specific antibody PY-20 (A). Alternatively, postnuclear supernatants were directly size-fractionated and analyzed by IB with phosphotyrosine-specific antibodies against LAT (pLAT, cross-reacting with pNTAL; B), pAkt

(C) or pErk (D). Total amounts of the proteins were detected by immunoblotting with protein-specific antibodies after stripping of the membranes. Relative amounts of tyrosine-phosphorylated proteins were determined by densitometry of the immunoblots and normalized to their levels in antigen-activated cells without vacuolin-1 (2 min) and total amount of proteins (Fold). The blots are representative of three independent experiments.

Fig 4 | Different effect of vacuolin-1 on Ca^{2+} uptake in RBL-2H3 cells and BMMCs. (A - C) Cells were treated with vehicle or 10 μM vacuolin-1 for 3 h and activated for 5 or 15 min as in Fig 2A - C, except that BSS-BSA was supplemented with 1 mM $^{45}Ca^{2+}$. The amount of cell-bound $^{45}Ca^{2+}$ was determined after centrifugation of the cells through 12% BSA gradient. Data represent means ± SD calculated from 3 experiments. Asterisks indicate significant differences.

Fig 5 | Vacuolin-1 inhibits actin polymerization in FcεRI-activated RBL-2H3 cells. RBL-2H3 cells and BMMCs were sensitized with IgE, treated with vehicle (black columns) or vacuolin (red columns) and activated through FcεRI for 2 or 8 min as in Fig 2A. The amount of F-actin was determined by flow cytometry. Data represent means ± SD calculated from 12 experiments. Asterisks indicate significant differences.

Fig 6 | Vacuolin-1 enhances membrane resealing after SLO permeabilization in RBL-2H3 cells. RBL-2H3 cells (A, summary in C) or BMMCs (B, summary in D) were treated with vehicle (in black) or 10 μM vacuolin-1 (in red) for 3 h and then treated with various concentrations of SLO from 0.4 U/ml to 4 U/ml in the presence or absence of Ca^{2+}. After removal of unbound SLO the cells were incubated for 50 min at 37°C, stained with PI and analyzed by flow cytometry. Percentage of PI-positive cells (means ± SD) was determined in 4-5 experiments. Asterisks indicate significant differences.

Table 1 | Effect of pharmacological inhibitors on cell viability and formation of vacuolin-1-induced vacuoles

Inhibitor	Main target of the inhibitor	RBL-2H3		BMMCs	
		LD_{50}* (μM)	MIC_{50}** (μM)	LD_{50} (μM)	MIC_{50} (μM)
IAA-94	Cl- channels	~200	>500	~200	>500
DIOA	K+/Cl- channels	~200	>500	~200	>500
NPPB	Cl- channels	~200	>500	~200	>500
DIDS	Cl- channels	~200	>500	~200	>500
Glybenclamide	K+/Cl-channels	~200	>500	~200	>500
Tamoxifen	PKC	~25	>500	~200	>500
Wortmannin	PI3K	>10	>10	>10	>10
PP2	Src kinases	>10	>10	>10	>10
Piceatannol	Syk kinase	>25	>25	>25	>25
Blebbistatin	NMM II ATPase	80	>120	80	>120
Bafilomycin A1	Vacuolar ATPase	>0.5	0.01	>0.5	0.01
ML-7	MLC kinase	>120	>120	>120	75
ML-9	MLC kinase	>120	>120	>120	75

*Lethal dose $(LD)_{50}$ indicates concentration of the drug decreasing cell viability by ~50%.

**Minimal inhibitory dose $(MIC)_{50}$ indicates concentration of the drug inhibiting formation of vacuolin-induced vacuolae by ~50%.

Supplementary Materials and Methods

Antibodies and reagents. The following mouse monoclonal antibodies were used: anti-FcεRI-β subunit [JRK, (Rivera *et al.*, 1988)], anti-LAT (Tolar *et al.*, 2001) and anti-dinitrophenyl (DNP)-specific IgE (IGEL b4 1) (Rudolph *et al.*, 1981)]. Horseradishperoxidase (HRP)-conjugated phosphotyrosine-specific mAb (PY20) was obtained from BD Biosciences (San Jose, CA, USA). Polyclonal antibody anti-phospho-LAT (specific for phosphorylated Tyr^{191}), which also reacts with NTAL was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-IgE antibody was prepared by immunization of rabbits with whole IGEL b4 1. Polyclonal antibodies specific for Erk, phospho-Erk (specific for phosphorylated Tyr^{204}), Akt1, phospho-Akt1 (specific for phosphorylated Ser^{473}) and HRP-conjugated donkey anti-goat IgG, goat anti-mouse IgG and goat anti-rabbit IgG, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories (Baltimore, PA, USA). Stem cell factor (SCF) and interleukin-3 (IL-3) were purchased from PeproTech EC, (London, England). Vacuolin-1 and PP2 were obtained from Calbiochem (La Jolla, CA, USA) and bafilomycin A1 was obtained from LC Laboratories (Woburn, MA, USA) or Sigma-Aldrich, Inc. (St. Luis, MO, USA). UltraLink-immobilized protein A was bought from Pierce (Rockford, IL, USA). FITC-labeled annexin V and $^{45}Ca^{2+}$ (sp. activity 566 MBq/mg) were obtained, respectively, from BD Biosciences and MP Biomedicals (Irvine, CA, USA). All other chemicals were from Sigma-Aldrich.

Cells and their activation. The origin of RBL-2H3 cells, and their culture conditions have been described elsewhere (Siraganian et al., 1982; Dráberová and Dráber, 1991). BMMCs precursors were isolated from femurs and tibias of C57BL.6J mice obtained from the Institute of Molecular Genetics (Prague, Czech Republic), and cultured in Iscove's medium supplemented with 10% fetal calf serum (FCS), SCF (40 ng/ml) and IL-3 (20 ng/ml). In the text, culture medium (CM) refers to Iscove's medium plus FCS. Sixteen to 18 h before the experiment the cells were transferred into CM with IL-3 and treated at the indicated time intervals with various concentrations of vacuolin-1 or DMSO alone (vehicle) in CM. In some experiments the cells were sensitized with TNP-specific IgE (IGEL b4 1 ascitic fluid) diluted 1:1000 in CM. Cells were harvested, washed with BSS (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM

$MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose) supplemented with BSA (1 mg/ml) and activated with antigen (TNP-BSA conjugate; 250 ng/ml). When the cells were activated with Ca^{2+} ionophore A23187 (1 μM) or thapsigargin (2 μM), the sensitization step was omitted. Mast cell exocytosis was assessed by measuring the relative content of β-glucuronidase released into supernatant. Aliquots of 20 μl of supernatant were mixed with 60 μl of 40 μM 4-methylumbelliferyl β-D-glucuronide and incubated for 60 min at 37°C. The reaction was stopped by adding 200 μl of ice-cold 0.2 M glycine buffer (pH 10.5), and fluorescence was measured in microtiter plate reader Fluorostar (SLT Labinstruments; Austria) at 365 nm excitation and 460 nm emission filters. The total content of β-glucuronidase was determined in supernatants from cells lysed by 0.05% Triton X-100. Cell size and viability were determined after trypan blue staining using Vi-Cell XR cell viability analyzer (Beckman-Coulter, Fullerton, CA, USA).

Inhibitors screening. RBL-2H3 cells and BMMCs were treated with various concentrations of inhibitors (see Table 1) for 30 min before adding vacuolin-1 (final concentration 10 μM). After further 3 h incubation with the inhibitor and vacuolin-1 cell viability was assessed by trypan blue staining. To evaluate the formation of vacuoles, cells were spun down (400 x g, 5 min), resuspended in 10 μl of fetal calf serum, smeared on microscopic slides and stained with May-Grünwald and Giemsa-Romanowski procedure. At least 100 cells were evaluated.

Immunoblotting. At indicated time intervals after activation, cells were pelleted and lysed for 30 min in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 1μg/ml aprotinin and 1μg/ml leupeptin, and supplemented with detergents 1% NP-40 and 1% n-dodecyl β-d-maltoside. After 30 min on ice postnuclear supernatants were resolved by SDS-PAGE and analyzed by immunoblotting with phosphospecific antibodies, followed by HRP-conjugated anti-mouse or anti-rabbit IgG antibodies. Alternatively, cells were solubilized in lysis buffer containing 0.2% Triton X-100, and IgE-FcεRI complexes from postnuclear supernatant were precipitated with rabbit anti-IgE polyclonal antibody prebound to UltraLink-immobilized protein A. Immunoprecipitated material was eluted with SDS-PAGE sample buffer, size fractionated and analyzed by immunoblotting with phosphotyrosine-specific antibody PY-20 conjugated to HRP. Immunoblots were developped with ECL and the signals were captured by Luminescent Image

Analyzer LAS 3000 (Fuji Photo Film Co, Tokyo, Japan) and further quantified and analyzed by AIDA image analyzer software (Raytest GmbH, Straubenhardt, Germany). The amount of tyrosine phosphorylated proteins was normalized to the amount of proteins immunoprecipitated as determined by densitometry of immunoblots after stripping of the membranes, followed by development with the corresponding protein-specific antibodies.

Flow cytometry analysis and F-actin assay. To determine the surface FcεRI expression, cells were exposed for 30 min on ice to 1μg/ml anti-TNP IgE followed by FITC-conjugated anti-mouse IgG cross-reacting with mouse IgE. Exposure of phosphatidylserine was detected by FITC-labeled annexin V as described (Smrz *et al.*, 2008). The amount of polymeric actin was determined by a modified previously described procedure (Howard and Meyer, 1984; Dráberová et al., 2003). Briefly, 10^6 cells in 200 μl BSS-BSA were stimulated or not with antigen for the indicated time intervals. The reaction was terminated by adding 300 μl of phosphate buffered saline, pH 7.4 (PBS) containing 50 μg of lysophosphatidylcholine, 6% formaldehyde and 0.125 μg/ml FITC-labeled phalloidin. After 10 min incubation at 37°C, the cells were centrifuged and resuspended in 200 μl of PBS before flow cytofluorometry analysis. Mean fluorescence intensities were determined by measurement in FL1 channel of FACSCalibur (BD Biosciences).

Uptake of extracellular calcium. Calcium uptake was determined by a modified procedure described previously (Dráberová, 1990). Briefly, IgE-sensitized or unsensitized cells ($2x10^6$) were resuspended in 100 μl BSS-BSA, mixed with equal volume of BSS-BSA supplemented with $^{45}Ca^{2+}$ and various activators, and incubated for 5 min or 15 min at 37°C. The reaction was terminated by placing the tubes on ice followed by suspending 100 μl aliquots on the wall of the 400 μl Beckman microtest tube separated by air space from 12% BSA in PBS (300 μl) at the bottom. Cell-bound $^{45}Ca^{2+}$ was separated from free $^{45}Ca^{2+}$ by centrifugation at 1200 x g for 15 min at 4°C through 12% BSA. Cell-bound radioactivity was recovered after freezing the tube, slicing off the tube bottom with pelleted cells into 1 ml of 1% Triton X-100, and solubilization of the cells for 12 - 16 h. Radioactivity was measured in 10 ml scintillation liquid (EcoLite, PM Biomedicals, Costa Mesa, CA, USA) in a scintillation counter with Quanta Smart software (PerkinElmer Life Sciences, Boston, MA, USA).

Plasma membrane repair assay. The assay was perfomed as described (Idone *et al.*, 2008) with some modifications. SLO and sodium dithionite, used for reduction of the SLO, were purchased from iTEST plus, Ltd. (Hradec Kralove, Czech Republic). A stock solution of SLO (10 U/ml) was prepared in PBS-BSA solution according to manufacturer's instructions by mixing SLO (22 U in 1.1 ml PBS-BSA) with 1.1 ml sodium dithionite in PBS-BSA. RBL-2H3 cells and BMMCs were treated with 10 μM vacuolin-1 or 0.1% DMSO (vehicle) in CM. After 3 h the cells were washed and their concentrations adjusted to $1x10^6$/ml in CM. Cells were distributed into U-shape 96-well plate (250 000 cells/well), centrifuged at 400 x g for 5 min at 4°C, resuspended in ice-cold BSS-BSA or Ca^{2+}-free BSS-BSA containing 1mM EGTA (BSS-BSA $–Ca^{2+}$) and kept on ice. An appropriate amount of SLO was then added and the cells were incubated on ice. 30 min later, cells were centrifuged as above and free SLO was removed. Pellets were resuspended in 250 μl BSS-BSA or BSS-BSA $–Ca^{2+}$ and incubated at 37°C for 50 min under continuous shaking. PI (1 μl from a stock 0.1μg/ml) was added and mixed, and at least 10 000 cells were evaluated by flow cytometry (FACSCalibur). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

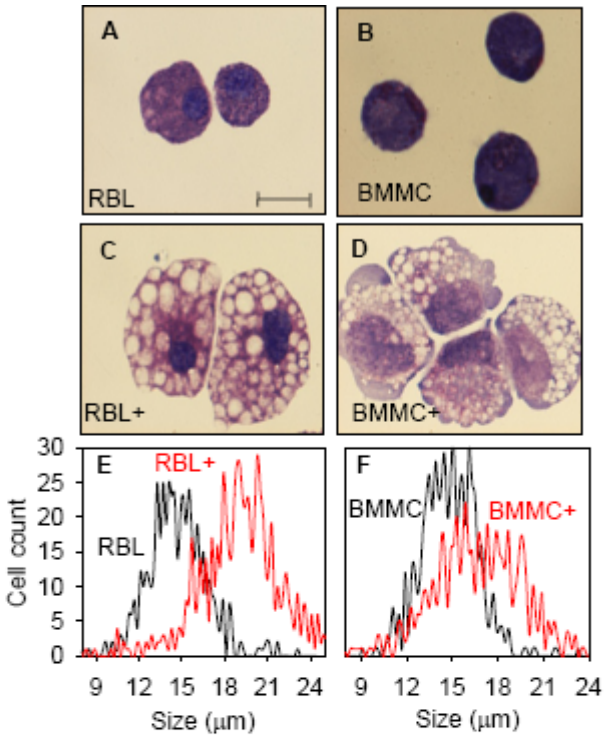

Fig. 1

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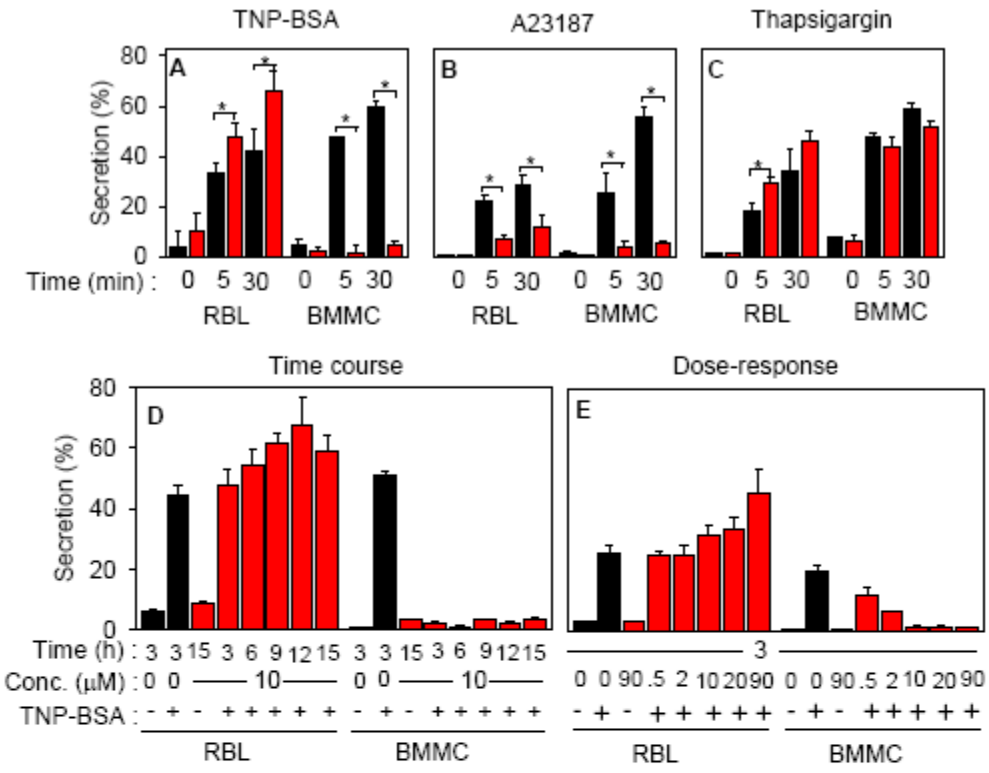

Fig. 2

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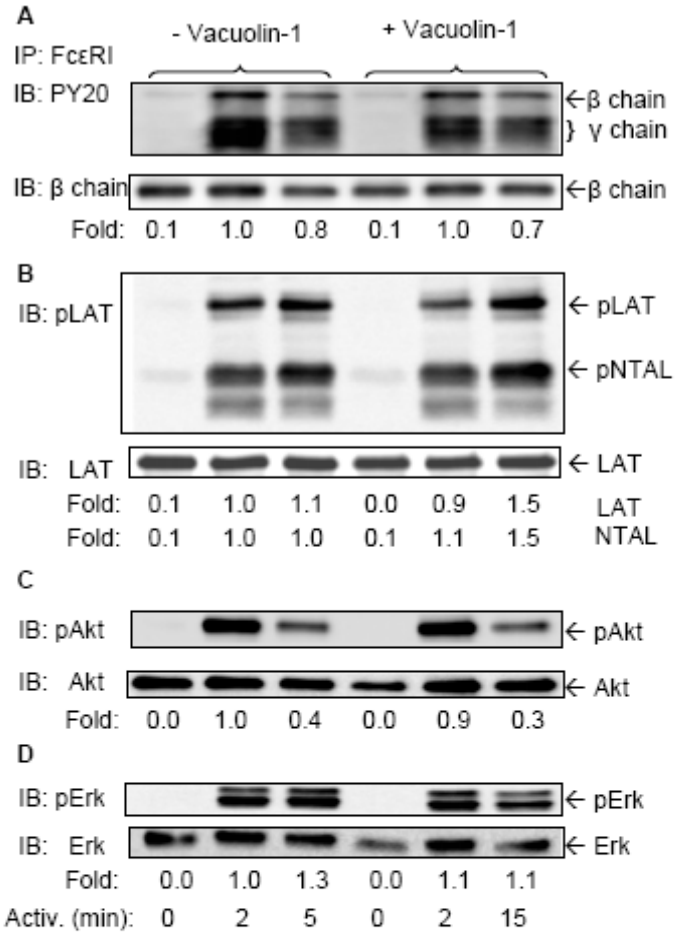

Fig. 3

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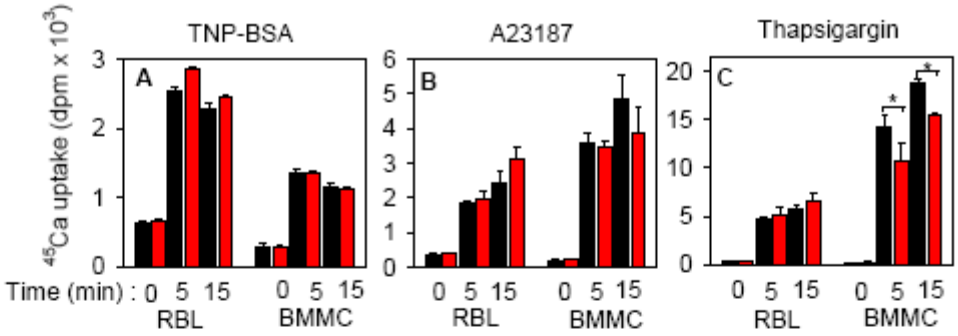

Fig. 4

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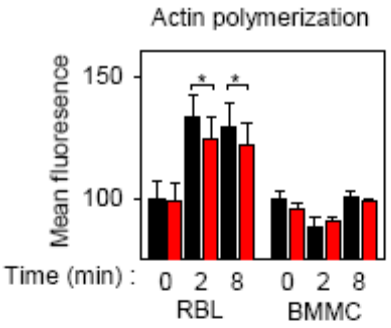

Fig. 5

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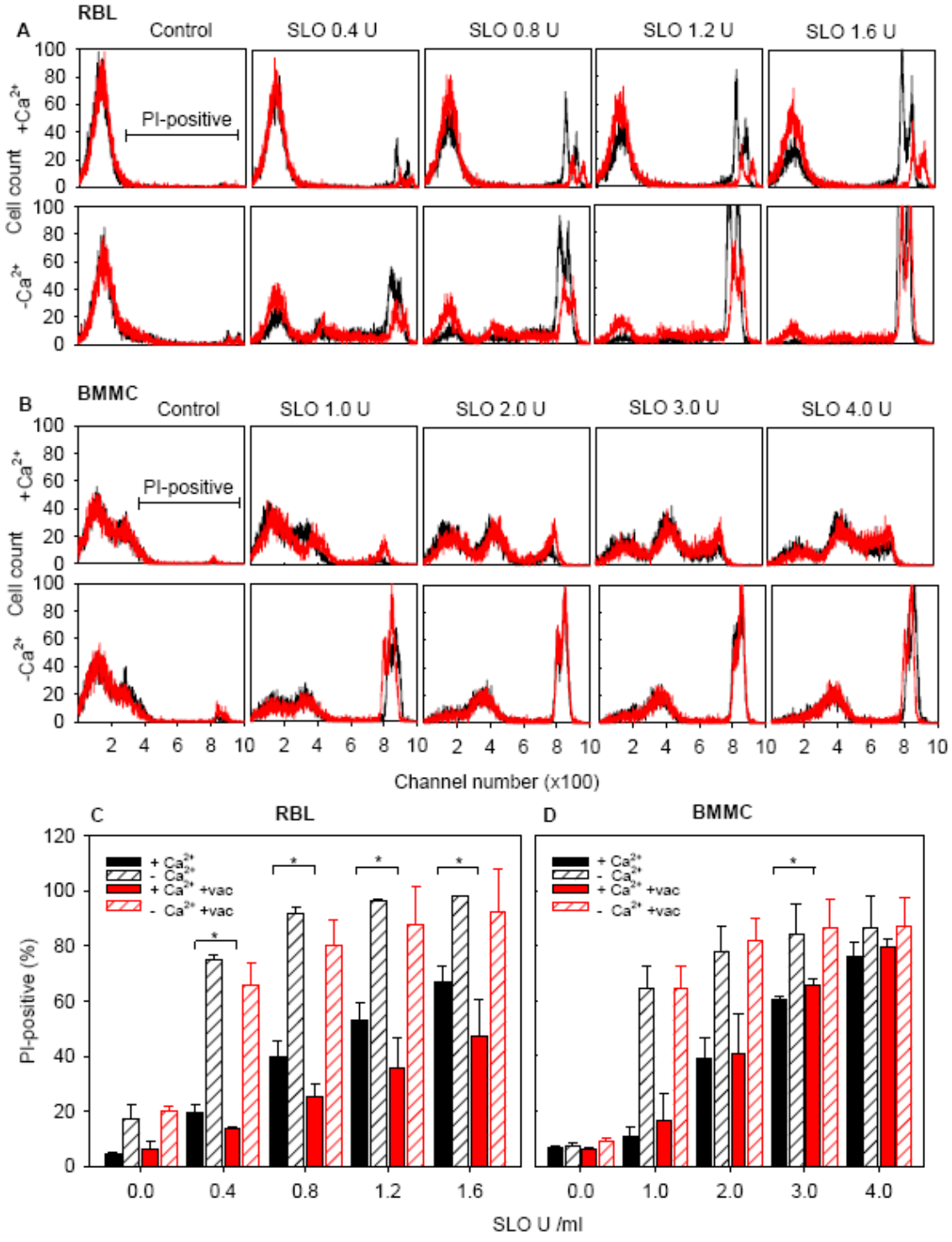

Fig. 6

Shaik et al.

4.3 Tetraalkylammonium derivatives as real-time PCR enhancers and stabilizers of the qPCR mixtures containing SYBR Green I

Nucleic Acids Res. 2008, 36: 1-10

The major aim of this study was to improve performance of SYBR green-based real-time PCR. To this aim we enriched PCR mixes with various additives and tested their effect on amplification in the presence of SYBR green 1. Being the first author of the paper I was involved in conceptual design of most of the experiments, performed most of them, except production of new monoclonal antibody specific for DNA polymerase, analysed data and participated in writing the manuscript.

4.4 Changes in GM1 ganglioside content and localization in cholestatic rat liver

Glycoconj J 2007, 24: 231-241

The major aim of this study was to localizate expression of GM1 ganglioside in hepatocytes of control and cholestatic rat liver. My contribution to this paper was to compare by PCR techniques the expression levels of genes GalTII and SATII in control and cholestatic rat liver cells. Being one of the authors I was involved in design of the corresponding experiments, analyzing my data and writing the corresponding paragraphs in Material and Methods and Results.

5. DISCUSSION

One of the major aims of this study was to contribute to the understanding the role of adaptor proteins NTAL and Grb2 in properties of mast cells. Permanent RBL-2H3-derived cell lines with low or enhanced expression levels of NTAL and/or Grb2 were prepared by RNAi or overexpression strategies. Even though RNAi can be achieved by direct transfection of target RNA into the cells, we repeatably failed with this approach and opted hairpin-mediated RNAi mainly because of it robustness and option to produce permanent transfectants. Unique targets of NTAL and Grb2 were cloned into mU6pro vector and transfected into RBL-2H3 cells. Upon expression the target hairpins are produced, leading to inhibition of expression of respective RNA. NTAL overexpression was achieved by cloning NTAL cDNA in to pcDNA3.1 vector. Variation of NTAL and/or Grb2 expression is shown to have multiple effects on FcεRI-mediated activation events.

Initial experiments revealed that RBL-2H3 cells with inhibited or overexpressed levels of NTAL showed decreased secretory response after FcεRI aggregation by antigen at all concentrations used. Flow cytometry showed no changes in the expression levels of FcεRI, excluding the possibility of reduced binding of IgE to the mutant cells.

Expression levels of NTAL affected morphology of the cells. Cells with enhanced NTAL levels were less adherent to tissue culture plates, whereas cells with less NTAL had longer processes. These differences could reflect NTAL-dependent changes in the amount of F-actin and its changes during FcεRI signaling. Cells with less NTAL had more F-actin compared to parental RBL-2H3 cells or cells with enhanced amount of NTAL.

Immunoblotting studies showed that inhibition of expression of NTAL did not inhibit the tyrosine phosphorylation of FcεRI β and γ subunits. Phosphorylation of other signaling proteins Syk and LAT was also not inhibited, but recruitment of PLCγ to signaling assemblies and its tyrosine phosphorylation and activation were inhibited leading to reduced production of IP3 in NTAL depleted cells (NTAL-). Low IP3 production led to a reduction in both the release of Ca^{2+} from intracellular stores and uptake of extracellular Ca^{2+} through store-operated calcium (SOC) missing in abbreviations channels. This could explain low secretion in Ag-activated cells.

Decreased tyrosine phosporylation of FcεRI subunits in NTAL overexpressors (NTAL+) suggested that the activity of Lyn kinase is reduced. However, immunocomplex kinase assay revealed that Lyn kinase activity in NTAL+ cells was not compromised implying that NTAL might interfere with the accessibility of Lyn to FcεRI. This possibility was further supported by data indicating that amount of Lyn coprecipitated with FcεRI is higher in RBL-2H3 than in NTAL+ cells. Lyn, like NTAL, have been proposed to be localized in lipid rafts (Brdicka et al., 2002; Dráberová and Dráber, 1993) and, therefore, it is possible that direct or indirect interaction of Lyn with NTAL prohibits the interaction between Lyn and FcεRI subunits.

Current study also indicates that different signaling assemblies are formed in RBL-2H3 cells depending on expression levels of NTAL. In NTAL+ cells more NTAL and less LAT is bound to Grb2, supporting the concept of competition between NTAL and LAT for Grb2 (Volna et al., 2004). Contrary to overexpressors in NTAL-deficient cells phosphorylation of FcεRI subunits, Syk and LAT was not inhibited. However, association of PLCγ with insoluble complexes, its phosphorylation and its activity was reduced which resulted in partial inhibition of downstream signaling. The impaired function of PLCγ could reflect involvement of NTAL in the formation of signaling assemblies required for PLCγ activity. As previously shown (Tailor et al., 1996; Volna et al., 2004) Grb2 forms complexes with SHP-2 phosphatase, and these complexes could bind to NTAL to regulate early signaling events. Alternatively, if PLCγ somehow interacts with NTAL its enzymatic activity would be inhibited in NTAL-deficient cells even though early signaling events are normal. If FcεRI signaling pathway is bypassed by thapsigargin, NTAL+ cells showed higher Ca^{2+} uptake from extracellular sources, suggesting NTAL could have a positive regulatory role in Ca^{2+} uptake. This would be in accordance with our finding of lower extracellular Ca^{2+} uptake by NTAL- cells. The exact role of NTAL in Ca^{2+} uptake is unclear but could be related to NTAL dependent Ca^{2+} regulating signal circuit described in DT40 B lymphocytes (Stork et al., 2004). In these cells Grb2 was shown to be a negative regulator in Ca^{2+} uptake, which appears to be eliminated upon binding to NTAL/LAT2.

However our evidence indicates that NTAL has different role in rodent mast cells. No inhibition of $[Ca^{2+}]_i$ was observed in BMMCs from $NTAL^{-/-}$ mice. Antigen-activated $NTAL^{-/-}$BMMCs showed no decrease but rather an increase in the uptake of extracellular $^{45}Ca^{2+}$. This observation

could be related to enhanced activity of PLCγ in NTAL$^{-/-}$ BMMCs (Volna et al., 2004; Zhu et al., 2004). Using RBL-2H3-derived cells we noticed that the highest Ca^{2+} response was observed in control RBL-2H3 cells where NTAL expression is probably kept at optimal levels, When NTAL level was decreased or increased, antigen-induced Ca^{2+} response was decreased. When the cells were activated by antigen in the absence of Ca^{2+} and then extracellular Ca^{2+} was added no dramatic differences between control RBL-2H3 cells and NTAL- was observed in calcium mobilization. In contrast the response in NTAL+ cells was inhibited. This inhibition reflected low levels of Ca^{2+} released from intracellular stores leading to low Ca^{2+} influx through SOC channels. Finally $[Ca^{2+}]_i$ is low in cells activated with thapsigargin in the absence of extracellular Ca^{2+} and this is independent of NTAL. But when Ca^{2+} was restored the increase in $[Ca^{2+}]_i$ correlated with amount of NTAL, suggesting positive regulatory role of NTAL in Ca^{2+} uptake via SOC channels.

Interestingly, no increase in tyrosine phosphorylation of NTAL was observed in thapsigargin-activated cells, suggesting novel regulatory role of NTAL independent of its phosphorylation. Predicted regulatory role of NTAL in FcεRI mediated activation is depicted in Fig. 5. At early stages of activation (phase I) NTAL serves as a substrate for protein tyrosine kinases and thus could interfere with phosphorylation of FcεRI and LAT by a competitive mechanism. Once phosphorylated NTAL binds Grb2 and other signaling molecules, which can modulate the activity of various enzymes like PI3 kinase and PLCγ. Enhanced activity of PLCγ leads to increased production of IP3 leading to increased cytoplasmic Ca^{2+} levels.

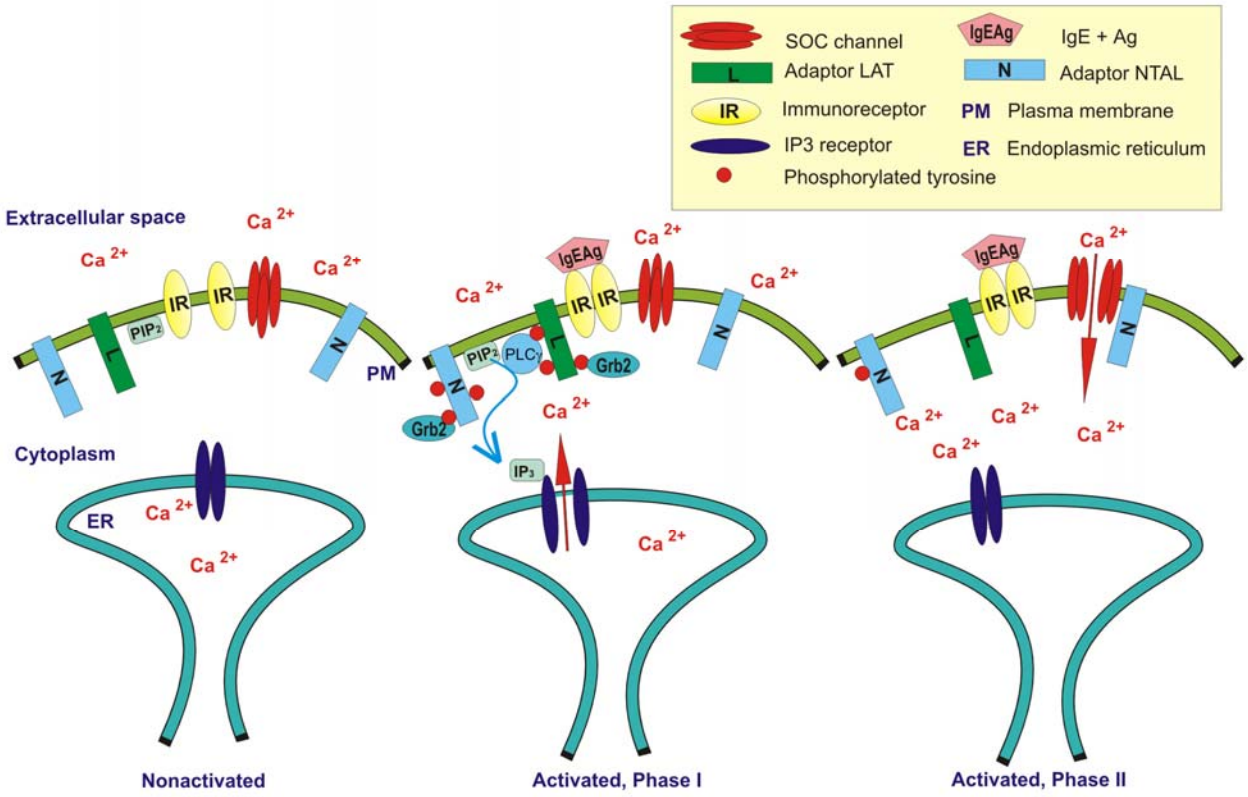

Fig.5. A model of NTAL function in FcεRI-mediated Ca^{2+} signaling. At early stages after FcεRI aggregation (phase I), NTAL is rapidly tyrosine phosphorylated, competing with phosphorylation of FcεRI and LAT. Phosphorylated NTAL binds Grb2 complexes and interferes with the activity of PI3K and several other signaling molecules. NTAL affects activity of PLCγ and in this way the generation of IP3 followed by release of Ca^{2+} from internal stores. At later stages of activation (phase II), extracellular Ca^{2+} flows into the cytoplasm through SOC channels. Activity of these channels could be modulated by direct or indirect interaction of NTAL with SOC channel proteins and/or regulators of their activity. However, this effect is independent of enhanced NTAL tyrosine phosphorylation.

At later stages of activation (Phase II), NTAL could interact with or indirectly affect the function of SOC channels. This could in part accomplished by direct or indirect interaction with SOC channel regulatory proteins Stim 1 and or Orai I (Feske et al., 2006; Liou et al., 2005; Putney, Jr. et al., 2001; Roos et al., 2005) and this function would be devoid of its phosphorylation state (Draber and Draberova, 2005). Predicted regulatory role of STIM1 in SOC is depicted in Fig.6.

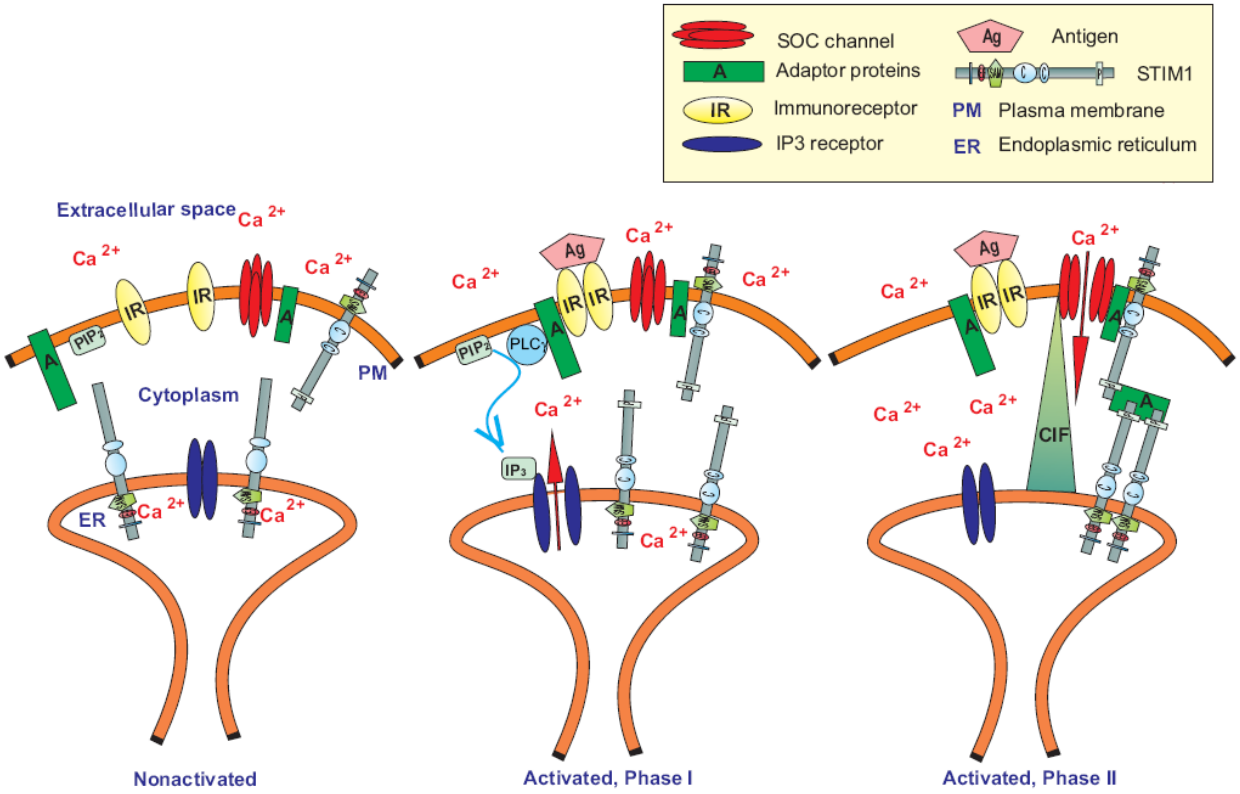

Fig. 6 A hypothetical model of STIM1 function in immunoreceptor signalling. The process of immunoreceptor signalling involves two phases of regulated increase in the concentration of free intracellular Ca^{2+}. Early signalling events involve antigen-mediated aggregation and tyrosine phosphorylation of the immunoreceptor subunits and other cellular proteins, including adaptors and PLCγ. Activated PLCγ cleaves PtdIns (4,5) P2 (PIP2) and produces Ins (1,4,5) P3 (IP3). Ins (1,4,5) P3 binds to the Ins (1,4,5) P3 receptor, a ligand-gated channel, which leads to a rapid and transient release of Ca^{2+} from the ER (phase I). The release of Ca^{2+} from the ER is sensed by the NH2-terminus of STIM1, which possesses the calcium-binding EF-hand motif. This causes the formation of STIM1 homoaggregates in the ER. The topographical changes in STIM1 lead to an alteration in SOC channels in the plasma membrane and increased flow of extracellular Ca^{2+} into the cytoplasm (phase II). The molecular mechanism of signal transfer from aggregated STIM1 to SOC channels is unknown. It can be speculated that communication of aggregated STIM1 in the ER with SOC channels could be mechanistically mediated by STIM1 in the plasma membrane and/or adaptor proteins. Alternatively, aggregated STIM1 in the ER could induce production of diffusible mediator CIF, resulting in SOC entry. (Draber and Draberova, 2005)

The model explained in Fig.5 is based on functional analysis of cells with enhanced or decreased expression of NTAL. These approaches even though useful have their own limitations. Knockdown approach may not always elucidate the function of target protein because of

compensatory activity of other proteins in place of target proteins. Overexpression approach which gives complementary data to knockdown approach also has its drawbacks of imposing unwanted effects by sequestering proteins they bind or by targeting proteins that are not their natural targets.

In summary, the combined data suggest that NTAL is a natural target of Lyn kinase and enhanced expression of NTAL could engage most of the Lyn kinase; this could explain decreased phosphorylation of FcεRI β and γ subunits and all downstream targets in FcεRI activated NTAL overexpressors. If NTAL by its interaction with Lyn kinase negatively regulates FcεRI phosphorylation one could expect to see enhanced FcεRI phosphorylation in activated NTAL- cells. However, we did not see such increase in this as well as other study (Volna et al., 2004); this could be explained by increased activity of phosphatases in such cells. Although activities of relevant kinases and phosphatases towards FcεRI could be higher in NTAL/LAT2 deficient cells compared to control RBL-2H3 cells, their functional equilibrium can be comparable.

Another major aim of this study was to elucidate the role of exocytosis in membrane repair in mast cells. To this end we used vacuolin-1, a small molecule that was shown to induce formation of large swollen structures derived from endosomes and lysosomes by homotypic fusion combined with uncontrolled fusion of the inner and limiting membranes of these organelles (Cerny et al., 2004). It has been reported that vacuolin-1 blocks the Ca^{2+}-dependent exocytosis of lysosomes induced by Ca^{2+} ionophore, or plasma membrane wounding (Cerny et al., 2004). Because resealing after wounding in vacuolin-treated cells was not affected, it was concluded that lysosomes are dispensable for resealing. This was unexpected finding because lysosomes had been known to undergo calcium-regulated exocytosis and because manipulations that inhibited lysosome plasma membrane fusions also inhibited resealing. Furthermore, when original experiments of Cerny's et al. were repeated, vacuolin-1, despite altering lysosome morphology, did not inhibit the exocytosis of lysosomes induced by exposure to Ca^{2+} ionophore or by plasma membrane wounding. In both these experiments authors used ionomycin for triggering exocytosis which is, however, not physiological inducer and has some side effects, including sharp decline in the ATP content of the cells as a result of increased plasmalemnal Ca^{2+} ATPase activity and the dissipation of mitochondrial proton gradient. These conflicting

studies led us to further examine the effect of vacuolin-1 on exocytosis induced by physiological activators in specialized secretory cells and compare the results with exocytosis induced by non physiological activators.

In our studies we observed that vacuolin-1 caused remarkable vacuolation of cytosolic compartments in both cell types, RBL-2H3 and BMMCs. There were dramatic morphological differences in cells treated with vehicle (DMSO) and vacuolin-1; for instance average diameter of the cells in suspension increased for more than 2 μm.

Further experiments showed that RBL-2H3 cells and BMMCs differ in their ability to secrete lysosomal contents after vacuolin-1 treatment. To check the ability of vacuolin 1-treated RBL-2H3 and BMMCs to secrete lysosomal contents, β-glucuronidase assay was performed. Both cells lines were activated via aggregation of FcεR1 receptors, ionophore, thapsigargin or VO_4. After activation at fixed time points supernatants were collected and β-glucuronidase assay was performed. Although all modes of activation led to secretion of lysosomal contents in both cell types, we saw dramatic differences in secretion after vacuolin-1 treatment. When activated via FcεRI receptors vacuolin-1 inhibited secretion in BMMCs and did not inhibit but rather potentiated exocytosis in RBL-2H3 cells. When activated via Ca^{2+} ionophore A23187, exocytosis in both cell lines was inhibited. In contrast when activated via SERCA ATPase inhibitor, thapsigargin, we didn't see significant differences before and after vacuolin treatment in both cell types. To explicate further, a dose-course of vacuolin-1 at a fixed time and a time-course with fixed concentration of vacuolin-1 was also performed. We found that vacuolin-1 affected RBL-2H3 and BMMCs in a very dose and time dependent manner. Interestingly, as short as 30 min of vacuolin-1 treatment at 10 μM concentration was enough to see perceptible differences in secretion. We thought that adherent state of RBL-2H3 cells may be the reason for the observed differences in secretion. To resolve this we cultivated RBL-2H3 cells in suspension, activated as above and found that they showed normal exocytosis.

In order to assign a molecular target and to decide whether formation of vacuoles depends on specific metabolic pathways we have screened effect of several pharmacological agents to nullify the effect of vacuolin-1. These inhibitors included Cl^- and K^+ channel blockers, protein kinase C (PKC) inhibitors, PI3 kinase inhibitor, Src and Syk kinase inhibitors and Myosin light chain

kinase inhibitors (ML-6, ML-7). None of the inhibitors showed any effect on vacuolin-1 induced vacuoles except bafilomycin, a specific inhibitor of vacuolar type H^+- ATPase (V-ATPase), which completely inhibited formation of vacuoles, indicating the origin of vacuoles involves the proton translocating activity of V-ATPases.

To see whether inhibition of secretion in BMMCs by vacuolin-1 treatment involves altered signaling pathways employed in FcεRI mediated activation, we observed phosphorylation of FcεRI β and γ subunits followed by phosphorylation of other key signaling proteins like LAT, NTAL/LAT2 Akt and Erk. We observed strong phosphorylation of these signaling proteins in both control and vacuolin-1 treated BMMCs indicating that early receptor-mediated tyrosine phosphorylation events are not affected by vacuolin-1 and are not responsible for the observed inhibition of secretion. We also checked cytoplasmic Ca^{2+} levels in control and vacuolin-1-treated cells. Both RBL-2H3 cells and BMMCs pretreated with vacuolin-1 or not were activated via FcεRI, Ca^{2+} ionophore or thapsigargin. We observed no significant effect of vacuolin-1 on extra-cellular Ca^{2+} uptake.

F-actin negatively regulates exocytosis (Frigeri and Apgar, 1999; Tolarová et al., 2004). We tested effect of vacuolin-1 on F-actin formation in activated and non activated RBL-2H3 and BMMCs before and after vacuolin-1 treatment. We observed in the case of RBL-2H3 cells activation via FcεRI induced enhanced formation of F-actin and a significant decrease in F-actin formation after treatment with vacuolin-1. This could explain the enhanced exocytosis of vacuolin-1 treated RBL-2H3 cells. In case of BMMCs no significant differences in F-actin polymerization was observed.

One of the processes by which cells overcome the plasma membrane injury is exocytosis and endocytosis. Both are Ca^{2+} and ATP-dependent processes (Idone et al., 2008). To check whether vacuolin-1-sensitive structures are involved in membrane resealing, we compared membrane resealing after bacterial toxin streptolysin O mediated injury in control and vacuolin-1 treated RBL-2H3 and BMMCs. When both cell types were treated with increasing concentration of SLO we observed an increased number of injured cells as determined by propidium iodide (PI) staining by flow cytometry. In case of RBL-2H3 cells pretreatment with vacuolin-1 resulted in a significant decrease in PI-positive cells, which goes in line with enhanced exocytosis. When

experiments were repeated in the absence of Ca^{2+} we saw increased number of PI-positive cells at given concentration of SLO compared to the presence of Ca^{2+} and vacuolin-1 has no significant effect on number of PI positive RBL-2H3 cells. These observations are in agreement with previous data showing that membrane repair is Ca^{2+} dependent in given cells (Walev et al and idone et al) and complement them by showing that vacuolin-1 promotes this process in RBL-2H3 cells.

BMMCs differ in their sensitivity to SLO; vacuolin-1 pretreatment did not enhance resealing after SLO treatment, but rather decreased it, as reflected by increased PI-positive cells. This is again in line with the observed inhibition of exocytosis by vacuolin-1. Number of PI-positive cells were high in Ca^{2+} free media, as expected, and vacuolin-1 had no significant effect on this parameter. This indicates that inhibition of exocytosis by vacuolin-1 in BMMCs has its counterpart to play in the membrane repair after SLO treatment.

This thesis had also specific aims in methodologies, such as construction of siRNA vectors (see above) and improving performance of PCR for routine analysis of gene expression. It has been previously reported that tetraalkylammonium (TAA) derivatives can serve as stabilizers of asymetrical cyanine dyes in aqueous solutions (Wu et al., 2002; Zeng et al., 1997). Because stability of dyes used for qPCR is problem, we decided to analyze whether wildly used cyanine dye SYBR green I could be also stabilized by TAA derivatives. Data obtained in this study indicate that TAA derivatives can be used as enhancers of real time PCR reactions employing SYBR green I. We also found that the effect of TAA derivatives varies with the nature of counter ions present in the reaction mixture and length of carbon chain of alkyl derivatives. Optimal results were obtained with 10-16 mM tetrapropylammonium chloride. Molecular mechanism of enhancing effect of TAA derivatives is unclear. Some of the possibilities can be considered. TMA-Cl is known to contribute towards thermal stability of AT base pairs, (Jacobs et al., 1988; Melchior, Jr. and Von Hippel, 1973). This could enhance base pair stability and thus contribute to more efficient binding of primers to template and result in efficient amplification. But our finding that, optimal annealing temperature was almost the same in control and TAA-Cl supplemented reaction mixtures suggests that contribution of these additives towards AT base pair stability was small. Previous studies showing base pair stability action of TAA-Cls was observed at very high concentrations up to 3 M, whereas enhancing effect of TAA-Cl (for

example TMA-Cl) was observed at relatively low concentrations, 16 mM. In fact higher concentration >32 mM was proven to be inhibitory to PCR.

Another possibility explaining the enhancing effect of TAA derivatives could be their interference with the inhibitory effect of SYBR green I on PCR. This possibility is supported by the finding that in the absence of SYBR green, template amplification evaluated by end point analysis showed similar yields of product in both control and additives-supplemented reactions. The structure of SYBR green I and its positive charges should allow its interaction with negative electrostatic potential in the minor groove of dsDNA. Moreover, van der Waals interactions within the boundaries of minor groove are likely to contribute to high affinity of SYBR green I towards dsDNA. These interactions could be affected by TAA derivatives. The highest fluorescence observed in reactions supplemented with tetrapentylammonium chloride (TPA-Cl) however had small effect on RT-PCR performance suggesting that, enhanced binding of SYBR green to the template inhibits rather than promotes PCR amplification. Interestingly, the observed enhancement of fluorescence in additives-supplemented reactions was decreased in samples supplemented with DMSO, suggesting DMSO could also contribute to the observed enhanced performance of PCR by partially reducing the binding of SYBR green I to the template. Yet these effects were small and could hardly explain the dramatic changes induced by DMSO and TAA-Cl additives.

Another important possibility worth considering is binding of SYBR green to single-stranded DNA (Zipper et al., 2004). In fact addition of single-stranded oligonucleotide primes to SYBR green I solutions showed significant increase in fluorescence. Addition of TAA-Cl derivatives inhibited such fluorescence and addition of DMSO further augmented this fluorescence inhibition. It can be argued that the enhanced fluorescence is a result of secondary structures of oligonucleotide primers and/or reflects formation of primer-dimers. To resolve this, we used poly $(T)_{20}$ and/or poly $(A)_{20}$ in reactions which cannot form any secondary structure and primer dimers. Interestingly, we still observed increased fluorescence, and addition of TAA-Cl and/or DMSO inhibited this fluorescence. Based on these findings we propose that SYBR green I can inhibit amplification through its binding to primers by discrete mechanism, one which interferes with their annealing and or initiation of PCR. This conclusion is supported by our finding that at limited concentration of primers amplification of electrophoretically detectable amplicons was

inhibited in the presence of SYBR green I. Of course our data do not exclude that SYBR green could inhibit PCR efficiency by some other mechanisms. In addition to these observations we consider important that TAA-Cl derivatives also contributed to stabilization of complete PCR mixtures (containing enzyme buffer and nucleotides). This could facilitate their handling at room temperature and storage and is at present in industry testing for preparing new generations of PCR mixtures.

Our optimized PCR master mixes were used in numerous experiments in department and also in collaborative studies. One of them is part of these studies because it helped to address an important issue how the expression of genes GalTII and SATII change in normal and cholestatic rat liver cells.

6. CONCLUSIONS

6.1 The role of NTAL and Grb2 in mast cell signaling

6.1.1 RNAi vectors were produced by cloning selected oligonucleotides specific for NTAL and Grb2 into mU6pro vector.

6.1.2 RBL-2H3 cell lines with decreased expression of NTAL and/or Grb2 were isolated after selection with geneticin.

6.1.3 RBL-2H3 cell lines were screened for decreased amount of NTAL and/or Grb2 by immunoblotting. Stable clones deficient in NTAL, Grb-2 or both NTAL and Grb2 were isolated.

6.1.4 We found that β-glucuronidase release was inhibited in RBL-2H3-derived cell lines with reduced expression of NTAL and/or Grb2, compared to control cells (transfected with empty vector or vector with mutated inserts).

6.1.5 We found decreased Ca^{2+} mobilization in NTAL deficient RBL-2H3 cells. We also find decreased uptake of Ca^{2+} after emptying endoplasmic reticulum stores with thapsigargin in the absence of extracellular Ca^{2+} indicating role of NTAL in SOC channel maintenance. We also found decreased Ca^{2+} mobilization in Grb2-deficient cells and it was further decreased in NTAL/Grb2 double-deficient cells.

6.1.6 We found that phosphorylation of early signaling proteins was not changed in NTAL-deficient RBL-2H3 cells, but late signaling events were inhibited.

6.1.7 We also found that reduced expression of NTAL had no effect on the surface expression on FcεRI. These data suggest that impaired response of RBL-2H3 cells with reduced expression of NTAL is not due to decreased binding of IgE-antigen complexes.

6.2 The role of mast cell exocytosis in membrane resealing

6.2.1 We found that treatment of mast cells with small chemical vacuolin-1 induces severe morphological changes represented by extensive formation of vacuoles and increased cell size in both RBL-2H3 cells and BMMCs.

6.2.2 We found that vacuolin-1-pretreated RBL-2H3 cells and BMMCs differ in their ability to secrete lysosomal contents when activated via FcεRI aggregation; exocytosis was enhanced in RBL-2H3 cells but inhibited in BMMCs. Exocytosis after

treatment with ionophore A23187 or thapsigargin also showed different sensitivity to vacuolin-1.

6.2.3 We have screened several pharmacological inhibitors, including K^+ and Cl^- channel blockers and found that only bafilomycin, an inhibitor of vacuolar H^+ ATPase, completely inhibited vacuolin-1-induced morphological changes. These data indicate involvement of proton translocating activity of H^+ ATPase in vacuolin-1-treated cells.

6.2.4 We found that mere vacuolin-1 treatment does not alter cell membrane integrity and does not induce apoptosis as confirmed by studying expression of FcεRI receptor and phosphatidylserine.

6.2.5 Effect of vacuolin-1 on membrane resealing after cell injury was determined. We developed new assay based on permeabilization of the cells with SLO, followed by resealing period and PI staining of damaged cells as determined by flow cytometry. We found that resealing of SLO-permeabilized cells differ in vacuolin-1-pretreated cells depending on capacity of vacuolin-1 to inhibit exocytosis; both exocytosis and membrane resealing in vacuolin-1-pretreated cells were enhanced in RBL-2H3 cells but were inhibited in BMMCs. These data supported hypothesis that lysosomal exocytosis is involved in membrane resealing.

6.3 Improving performance of PCR by modifying composition of PCR mixes

6.3.1 We identified TAA derivatives as additives of PCR enhancing amplification efficiency, reducing non-specific amplification and enhancing PCR performance even after prolonged storage at suboptimal temperatures. Optimal results were obtained with PCR mixes supplemented with 10-16 mM tetrapropylammonium chloride.

6.3.2 We formulated and used new PCR mixes for analysis of expression levels of selected genes. We found e.g. that expression levels of GalTII and SATII were the same in control and in cholestatic liver cells. This finding contributed to formulation of new hypothesis that not only amount of gangliosides but also their topography is important for progress in cholestasis. New PCR mixes were extensively used for routine screening of mast cells isolated from normal and NTAL- and/or LAT-deficient mice to confirm their genotype.

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